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THE ROLE OF CYTOKINES IN THE PATHOGENESIS OF INFLAMMATORY EYE DISEASE

Denis Wakefield* and Andrew Lloyd

A coherent view of the role of cytokines in inflammatory eye disease is emerging as a result of studies both in man and experimental animals. Cytokines have been demonstrated in ocular tissue obtained from patients with intraocular inflammation (uveitis) (gamma interferon, IL-2) and have been shown to induce inflammation in experimental animals after intraocular injection [(IL-1, IL-6, IL-8, tumour necrosis factor (TNF), granulocyte macrophage-colony stimulating factor (GM-CSF)]. Several unique features of the immunology of the eye such as the immunosuppression associated with anterior chamber associated immune deviation (ACAID) may be due to the effects of cytokines. Similarly, common complications of ocular inflammation such as glaucoma, keratic precipitates, retinal (macular) oedema and neovascularization may be mediated by cytokines. Understanding of the role of cytokines in inflammatory eye disease has the potential to lead to the development of therapies to abrogate the effects of these important mediators of the inflammatory response.

Inflammation of the eye is a common clinical problem that may involve any part of the eye. The uvea, the middle coat of the eye, takes the brunt of most serious ocular inflammation as a result of its extreme vascularity. Uveitis is a complex group of diseases resulting from diverse aetiologies and pathogenic mechanisms, the study of which has provided increasing insight into the role of cells and biologically active molecules in the immunopathogenesis of these diseases. Uveitis is a leading cause of visual impairment and blindness in most countries.^{1,2} It is characterized by acute, recurrent or persistent inflammation that may effect the anterior (anterior uveitis, AU) or posterior uvea (posterior uveitis, PU). Despite extensive investigation, uveitis remains an idiopathic disease in the vast majority of

cases.^{1,2} Recent evidence from studies in human uveitis and animal models, including experimental autoimmune uveitis (EAU) induced by several different retinal antigens, and endotoxin-induced uveitis (EIU) induced by lipopolysaccharide (LPS), has significantly increased our knowledge of the pathogenesis of ocular inflammation.

There is considerable experimental evidence for the concept that T cells and cytokines play a major role in the pathogenesis of uveitis. First, T cells have been demonstrated to be the most abundant cell type in the uveal tissue, retina, aqueous and vitreous humor of patients with uveitis.³ T cells are also the principle cells implicated in the pathogenesis of retinal-S antigen-induced EAU.⁴ Adoptive transfer experiments have revealed that T lymphocytes, and not serum, can transfer this disease.⁴ Inflamed uveal tissue demonstrates increased HLA class I and class II antigen expression, probably induced by cytokines.⁵⁻⁷ Interleukin 2 (IL-2) has been detected in inflamed uveal and retinal tissue and IL-2 receptor levels are increased in patients with certain types of uveitis.⁸ Finally, the dramatic suppressive effect of cyclosporin-A in controlling uveitis has been repeatedly demonstrated in EAU and human disease.^{9,10} This immunosuppressive drug is known to inhibit the synthesis of several cytokines, particularly

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IL-2 and interferon (IFN) gamma.¹¹ It is likely that the secretory products of T cells and other cytokines play an important role in the pathogenesis of uveitis.

Evidence for the role of cytokines in the pathogenesis of uveitis comes principally from: (a) detection of cytokines in ocular tissue or fluid, (b) in-vivo studies in experimental animals and (c) in-vitro studies such as MHC antigen expression.

INTERLEUKIN 1

Evidence of an acute phase response in patients with uveitis indicates the likelihood of increased IL-1 (or tumour necrosis factor (TNF) and/or IL-6) activity in such patients.¹² Intravitreal injection of recombinant IL-1 into the rabbit or rat eye leads to the development of a severe uveitis.^{13,14}

The relative concentration of IL-1 in ocular fluids and the in-vivo production of IL-1 by ocular cells and infiltrating inflammatory cells in the human eye has not been ascertained. Helbig and colleagues¹⁵ recently reported the effects of endotoxin on the production of inflammatory mediators by cultured bovine pigmented ciliary epithelial cells. Using bioassays they found membrane-associated IL-1 activity in response to endotoxin, but no TNF activity was detected.

Two groups have reported that IL-1 is a potent angiogenic factor, an observation of considerable relevance to ocular disease where neovascular proliferation is a common manifestation.^{16,17}

INTERLEUKIN 2

Hooks et al.⁸ demonstrated the presence of IL-2 in the cytoplasm of lymphocytes infiltrating the choroid of patients with sympathetic ophthalmia. Recently, Rosenbaum et al.¹⁸ have reported increased IL-2 receptor levels in the serum of patients with a variety of uveitis syndromes including: sarcoid uveitis, pars planitis and bilateral chronic anterior uveitis. It is highly likely that IL-2 is involved in ocular immune responses, especially in view of the presence of activated T cells in the uvea and peripheral blood of patients with active uveitis and its crucial role in T cell-mediated immunity.

INTERLEUKIN 6

IL-6 appears to be an important mediator of the inflammatory response and several studies indicate its potential role in inflammatory eye disease. Koekzema and Kijlstra¹⁹ examined the serum and aqueous humor concentrations of IL-6 in rats with endotoxin-induced uveitis, and rats made tolerant to endotoxin. Using a bioassay, they found elevated levels of IL-6 in the serum and aqueous humor of these animals. Furthermore, they have shown that intravitreal injection of

IL-6 into both rats and rabbits produces uveitis. This same group have recently detected IL-6 in the aqueous humor of patients with heterochromic cyclitis, a chronic idiopathic form of anterior uveitis often complicated by glaucoma in which the iris loses pigment and undergoes atrophy (personal communication). The cellular source of IL-6 in the eye has not been ascertained, although it is likely that a variety of resident ocular cells and inflammatory cells are responsible for its production.

TUMOUR NECROSIS FACTOR

Bando et al.²⁰ have extensively studied the time course of appearance of TNF- α in endotoxin-induced uveitis (EIU). The observed leukocytosis and increased protein concentration in the aqueous humor correlated with the pattern of increased serum TNF- α levels.

Rosenbaum et al.¹³ examined the effects of intravitreal injection of TNF, IL-1 and endotoxin in a rabbit model of uveitis. They found that recombinant human TNF- α produced inconsistent and mild inflammatory changes in the iris and ciliary body, that were less marked than those produced by IL-1 or endotoxin. There have been no reports of the detection of TNF- α in the serum or aqueous of patients with uveitis.

INTERFERON GAMMA

Interferon gamma (IFN- γ) also appears to play an important role in the pathogenesis of uveitis. In patients with uveitis there is increased expression of both class I and class II HLA antigens on the cells of the iris,^{6,7} retinal pigment epithelium and vascular endothelium.⁸ It has been shown that the degree of enhanced expression of these HLA antigens on the iris is directly correlated with the concentration of IFN- γ in the aqueous humor.⁷ The concentration of IFN- γ is not increased in the serum of most patients with anterior uveitis,²¹ although it has been shown to be increased in the serum of patients with Behcet's syndrome,²² a systemic inflammatory disease involving multiple organs associated with severe uveitis. Patients with anterior uveitis have been shown to have increased serum neopterin levels reflecting systemic IFN- γ activity.²¹

Human uveal cells, with the exception of vascular endothelium, normally express little or no class I HLA antigens in vivo.^{6,7} These antigens are inducible on uveal cells in vivo by cytokines, especially IFN- γ . Abi Hanna and Wakefield^{23,24} have examined the effects of a number of different cytokines, and combinations of cytokines, for their effect on HLA antigen expression on uveal cells. These experiments revealed that IFN- γ is the most potent inducer of class I and class II HLA antigen expression.^{24,25} Combinations of IFN- γ with TNF- α and TNF- β produced an enhancement of class

I antigen which was more than the additive effect of the two cytokines alone. Moreover, it has been shown that iris cells in biopsies from patients with anterior uveitis (AU), and retinal pigment epithelium from patients with posterior uveitis, but not from patients with senile cataract or normal eyes, express class I HLA antigens *in vivo*.⁶ These observations, and those of others, suggest that uveal inflammation may only be possible following HLA antigen induction on specific target tissues, and that IFN- γ , along with other cytokines, may be involved in this process.

Further evidence for the role of IFN- γ in uveitis is provided by studies demonstrating that intravitreal injection of this cytokine into the eyes of experimental animals enhances the production of EAU induced by retinal antigens such as interphotoreceptor retinoid-binding protein (IRBP). Recently, Atalla et al.²⁶ have shown that the systemic injection of antibodies to IFN- γ in animals, prior to the induction of EAU, ameliorates this disease. In contrast, Caspi et al.²⁷ have shown that antibodies to IFN- γ can enhance the development of EAU. Thus, IFN- γ appears to be an important cytokine in the induction and perpetuation of uveal inflammation in patients with inflammatory eye disease.

INTERLEUKIN 8

Ferrick et al.²⁸ have recently shown that the intravitreal injection of IL-8 into the eyes of experimental animals is capable of inducing an acute inflammatory response. However, the role of this cytokine in human disease is yet to be studied.

COLONY STIMULATING FACTORS

Rosenbaum et al.²⁹ have demonstrated that the intravitreal injection of granulocyte macrophage-colony stimulating factor (GM-CSF) is capable of inducing uveitis in the rabbit eye. It is interesting to note that this inflammatory response is also associated with a decrease in intraocular pressure. Thus GM-CSF may be involved in the pathogenesis of uveitis and possibly the regulation of intraocular pressure, although its role in human disease has not yet been clearly ascertained.

TRANSFORMING GROWTH FACTOR BETA

TGF- β is potentially one of the most interesting cytokines in relation to inflammatory eye disease. Cousins et al.³⁰ have shown that this cytokine is present in the aqueous humor of normal non-inflamed eyes. Streilein's group³¹ have presented fascinating data indicating that TGF- β may be intimately involved in the anterior chamber associated immune deviation

(ACAID) phenomenon, whereby injection of tumours or other antigens into the anterior chamber of the eye will lead to a suppression of the cell mediated immune response to these antigens after systemic challenge. The cause of this suppressed immune response is unknown, although recent studies indicate that one of the factors that may be involved in this immune suppression is TGF- β .³⁰ It is tempting to postulate that this inhibitory cytokine may also play an important role in other immune responses within the eye, particularly related to the control of the inflammatory response in uveitis. Thus far there have been no studies of this cytokine in human uveitis.

It is possible that a variety of other cytokines play an important role in the pathogenesis of inflammatory eye diseases. There is also evidence that certain cytokines are probably not important in the pathogenesis of these diseases. Rosenbaum et al.²⁹ have shown that the intravitreal injection of IL-3 or GM-CSF into the vitreous of the eyes of experimental animals do not induce uveitis. There are no data available on the role of these cytokines in human disease. A theoretical model of the role of cytokines in the induction of uveitis is outlined below.

HYPOTHETICAL ROLE OF CYTOKINES IN THE PATHOGENESIS OF UVEITIS

Although the cause of most cases of uveitis remains unknown, it is believed that the disorder is precipitated by exogenous or endogenous antigens. Following antigenic stimulation, a localized immune response is generated in the eye. The initial event probably involves antigen processing and presentation by antigen-presenting cells (APCs). Whether this is mediated by endogenous ocular cells or infiltrating mononuclear cells has not been established, although it is known that a number of uveal and retinal cells can function as APCs including RPE, and uveal fibroblasts (Abi Hanna and Wakefield, unpublished observation). Retinal glial cells (Muller cells) have been shown to profoundly suppress antigen activation, as well as IL-2 dependent expansion of T cells, through a contact dependent mechanism. As the retina is often involved in severe uveitis it is possible that organ resident, non-lymphoid cells such as the Muller cell, may play a role in the control of local immune responses in the eye.³²

In a typical T cell-mediated immune response the triggering antigen is processed by the APCs and the antigenic peptide is bound to major histocompatibility complex (MHC) molecules, on the cell surface, where it is recognized by T cells possessing antigen-specific receptors. Once activated these T cells secrete cytokines that stimulate and amplify the immune response.

An initial event in the ocular inflammatory response is exudation of proteinaceous material from dilated blood vessels followed by the migration of

inflammatory cells into the eye. In order for this to occur leucocytes must first adhere to the vascular endothelium. Thus, within the inflamed uvea, increased adhesiveness of the post capillary venules for circulating polymorphonuclear leukocytes and mononuclear cells is probably an early event and may be due to the action of cytokines such as IL-1, TNF- α and IFN- γ on the induction of adhesion molecules (e.g. ICAM-1/ELAM) on the uveal endothelial cells.³³ The subsequent chemotaxis of these adherent cells into the uvea is most likely under the influence of a number of factors, including IL-8. The expression of class II MHC antigens by endothelial cells and other cells of the inflamed uvea is stimulated by IFN- γ (and possibly GM-CSF). It may be that induction of MHC antigens on uveal cells is one of the earliest events in uveitis and this change may result in an increased potential for antigen presentation and amplification of the ocular immune response.³⁴

Activation and proliferation of ocular T cells, B cells and macrophages, is likely to be primarily related to the actions of IL-2, although IL-1, IL-6 and TNF- α may amplify these responses. The differentiation of these cells is primarily influenced by IL-2, with IL-1 and IL-6 perhaps also playing a part. There is accumulating evidence that IL-6, together with IL-1 and TNF- α are the most potent and important cytokines in local and systemic inflammatory responses. Macrophages are activated at the site of the inflammatory response by IFN- γ , GM-CSF and IL-2.

Uveitis is characterized by a number of unique features that may be explicable in terms of the activity of specific cytokines. In severe uveitis and in recurrent uveal inflammation, iris atrophy is often a striking feature. The cause of this is unknown, although it is possible that cytokines such as IFN- γ , TGF- β and IL-6, which inhibit fibroblast proliferation *in vitro*, may play a role. The effects of such cytokines on uveal fibroblast proliferation has not been examined. One of the characteristic clinical signs of uveitis is the appearance of keratic precipitates (KP) on the endothelial surface of the cornea. Despite the fact that a variety of cell types have been reported to be present in such KP the pathogenesis of these lesions is completely unknown. We hypothesize that they are the result corneal endothelial cell (CE) activation by cytokines (e.g. IFN- γ , TNF- α , IL-1) released into the aqueous humor during uveitis. Such cytokines would be expected to activate the CE causing it to express adhesion ligands for leucocytes. Neovascularization, a common feature of the inflamed uvea may occur in response to IL-1, TNF- α , fibroblast growth factor (FGF), epidermal growth factor (EGF), TGF- β , GM-CSF and platelet-derived growth factor. Oedema of the macula, which is a common, sight-threatening complication of chronic uveitis, may be due to a

TABLE 1. Possible role of cytokines in uveitis.

Clinical features of uveitis	Possible cytokines involved
Increased vascular permeability, macula oedema, aqueous flare	IL-1, TNF, INF- γ
Expression of vascular and corneal adhesion molecules	TNF, IL-1
Lymphocyte infiltration and activation	IL-1,2,4,6,8, TNF IFN- γ
Macrophage infiltration and activation	IL-1,2,4,6, TNF IFN- γ , GM-CSF, M-CSF, MCAF
HLA antigen expression	IFN- γ , IL-6, TNF
Angiogenesis	IL-1, TNF, PDGF, TGF- β , FGF, PGF
Iris atrophy and lack of fibroblast proliferation	IL-6, IFN- γ
Changes in intraocular pressure, glaucoma, hypotony	GM-CSF, IL-1, TNF IFN- γ

number of cytokines that increase vascular permeability, including IFN- γ and TNF- α . Glaucoma, a frequent complication of uveitis, may also be promoted by cytokines that stimulate production of aqueous humor or impede aqueous drainage. Similarly, hypotony, the low pressure found in end stage blind eyes of chronic uveitis may be promoted by cytokines such as GM-CSF. The possible role of cytokines in uveitis are summarized in Table 1.

A number of cytokine inhibitors, such as TGF- β , and the recently described IL-1 inhibitory molecules, may also play a modulatory role at the site of inflammatory responses within the eye.

A coherent view of the role of cytokines in uveitis is emerging as a result of studies both in man and experimental animals. Several unique features of the immunology of the eye such as ACAID may be due to the effects of cytokines. Understanding of the role of cytokines in uveitis has the potential for the development of therapies to abrogate the effects of these important mediators of the inflammatory response.

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Matrix Metalloproteinases and Tumor Necrosis Factor α in Glaucomatous Optic Nerve Head

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Objective: To study expression and location of matrix metalloproteinases (MMPs) and tumor necrosis factor α (TNF- α) in glaucomatous optic nerve heads, which are known to be secreted in response to a variety of neuronal injury.

Methods: Four postmortem eyes from patients with primary open-angle glaucoma, 7 eyes from patients with normal-pressure glaucoma, and 4 eyes from age-matched normal donors were studied by immunohistochemistry. The sections of the optic nerve heads were examined after immunostaining with antibodies to MMPs (MMP-1, MMP-2, and MMP-3), TNF- α , or TNF- α receptor 1.

Results: The intensity of the immunostaining and the number of stained cells for MMPs, TNF- α , or TNF- α receptor 1 were greater in the glaucomatous optic nerve heads, particularly in eyes with normal-pressure glaucoma compared with age-matched controls. Positive immunostaining was observed in all regions of the glauco-

matous optic nerve heads, but most prominently in the postlaminal region. Immunostaining was observed mainly in glial cells and their processes around the axons and blood vessels and in pial septae.

Conclusion: There is increased immunostaining for MMPs, TNF- α and TNF- α receptor 1 in the glaucomatous optic nerve head, which suggests increased expression of these proteins in glaucoma and thereby implies a role in the tissue remodeling and degenerative changes seen in glaucomatous optic nerve heads.

Clinical Relevance: The MMPs and TNF- α may be components of astroglial activation that occurs in glaucomatous optic nerve heads. The biological alterations in the expression of these proteins may play a role in the progression of glaucomatous optic neuropathy.

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HISTOPATHOLOGIC studies of the glaucomatous optic nerve head in primary open-angle glaucoma (POAG) reveal astroglial activation and tissue remodeling, which accompanies neuronal damage. As a part of tissue remodeling, backward bowing and disorganization of the lamellar cribriform plates are common characteristics of glaucomatous eyes with either normal or high intraocular pressure.¹ These histologic changes are accompanied by the up-regulation of extracellular matrix components, including collagen and proteoglycan, and adhesion molecules by optic nerve head astrocytes in glaucomatous eyes.²⁻⁶ The astroglial activation seen in glaucomatous optic nerve heads likely represents an attempt to limit the extent of the injury and promote the tissue repair process. However, despite the astroglial activation, there is limited deposition of extracellular matrix in glauco-

matous optic nerve atrophy, which does not retain characteristics of scar tissue formation.^{7,8} This suggests that there are diverse cellular responses to the initial event or subsequent tissue injury, which preferentially results in tissue degradation.

Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade components of extracellular matrix. Increased secretion of MMPs by activated glial cells have been implicated in various extracellular matrix remodeling events that occur during normal development and in a number of pathologic processes, including atherosclerosis, arthritis, tumor growth, and metastasis.⁹⁻¹³ In addition, reactive astrocytes after neuronal injury produce various neurotrophic factors and cytokines, including tumor necrosis factor α (TNF- α),¹⁴ which play a critical role in the regulation of the synthesis of MMPs.¹⁵⁻¹⁷ Furthermore, the release of TNF- α from its membrane-bound precursor is a MMPs-dependent process.¹⁸

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PATIENTS AND METHODS

PATIENTS

Four postmortem human eyes with a diagnosis of POAG and 7 human eyes with a diagnosis of NPG were obtained. The age of patients ranged from 68 to 84 years. **Table 1** outlines the clinical findings that were available from glaucomatous eyes. Four human donor eyes with no history of eye disease were used as age-matched controls (age range, 61-81 years). The death-to-fixation time for the specimens ranged between 6 and 9 hours.

IMMUNOHISTOCHEMISTRY

After enucleation, all eyes were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in graded alcohol, and embedded in paraffin. Since some of the specimens contained only the optic nerve head and small portions of the peripapillary retina, retinal distribution of immunostaining was not studied. After deparaffinization, 5- μ m-thick longitudinal sections of optic nerve heads were incubated with monoclonal antibodies against MMP-1, MMP-2, or MMP-3 (2.5 μ g/mL) (Oncogene Science, Cambridge, Mass) or polyclonal antibodies against TNF- α or TNF- α receptor 1 (2 μ g/mL) (R & D Systems, Minneapolis, Minn) overnight at 4°C, after endogenous peroxidase was blocked with 2% hydrogen peroxide in methanol and followed by several washes in phosphate-buffered saline solution. The 3 anti-MMP antibodies recognized both latent as well as active forms of MMPs. Prior to incubation with primary antibodies, the sections were incubated with either mouse skin extract (during MMP staining) or 20% nonimmune donkey serum (during TNF- α and TNF- α receptor 1 staining) for 20 minutes

to block background staining. Biotinylated secondary antibody (anti-mouse or anti-goat IgG) (Dako Corp, Carpinteria, Calif) was applied to the sections for 30 minutes at room temperature. The slides were then incubated with horseradish peroxidase-labeled streptavidin solution (Dako Corp) for 30 minutes, and the reaction was visualized by incubation in a solution of 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.006% hydrogen peroxide in 0.05M Tris-HCl (pH, 7.6). The slides were lightly counterstained with Mayer hematoxylin. Sections incubated with mouse serum or phosphate-buffered saline solution in place of the primary antibody served as negative controls. Sections from biopsy specimens of infiltrating ductal breast carcinoma served as a positive control for all antibodies used in this study.

Three to 5 sections from each optic nerve head were examined by immunohistochemistry for each protein including MMPs, TNF- α , and TNF- α receptor 1. To obtain comprehensive semiquantitative evaluation of the immunostaining, the intensity of immunostaining for MMPs and TNF- α and its receptor in the prelaminar, laminar, and postlaminar regions of the optic nerve head was graded using an arbitrary score in which each region was graded from - to +++. A semiquantitative score (indicated in parentheses) was then calculated for each optic nerve head (-, absent [0]; +, ranging from absent to weak [0.5]; ++, moderate staining [1]; +++, strong staining [3]). The grading of the immunostaining was performed in a masked fashion by an observer who was skilled in grading immunohistochemical staining but was not familiar with the pathologic changes in the optic nerve head. The observer graded the intensity of immunostaining in optic nerve head regions (prelaminar, laminar, and postlaminar) that were pointed out by one of the authors (X.Y.). Both the scored results and the photographs of representative sections from each group are presented.

Recently, we reported the histopathologic features of a pair of eyes with normal-pressure glaucoma (NPG) that demonstrated Schnabel optic atrophy.¹ In addition to the eyes presented in the report, our histopathologic examinations in an additional 5 postmortem eyes from patients with NPG revealed Schnabel cavernous atrophy. Like eyes with POAG, the eyes with NPG exhibited bowing of the lamina cribrosa, areas of glial activation, and axonal atrophy. However, the remarkable histopathologic distinctions between the eyes with NPG or POAG were the large cavernous spaces in the optic nerve head, extensive loss of pial septae, and the absence of a fibroglial response. These findings suggested that one possible mechanism for the cavernous degeneration might be an exaggerated astroglial response to degrade extracellular matrix by proteolytic enzymes such as the MMPs.

In this immunohistochemical study, antibodies against MMPs (MMP-1, MMP-2, and MMP-3), TNF- α , and TNF- α receptor 1 were used to label optic nerve head sections from postmortem eyes with POAG or NPG and from age-matched normal donors. Our observations suggest that there is increased expression of MMPs, TNF- α , and TNF- α receptor 1 in the glaucomatous optic nerve head, particularly with NPG, which likely contributes to the tissue remodeling seen in glaucomatous optic neuropathy.

RESULTS

The normal eyes exhibited glial columns and nerve bundles in the prelaminar region when observed by light microscopy. In the lamina cribrosa, there were glial cells lining the collagenous laminar beams; in the postlaminar region, the glial cells were mainly distributed along the pial septae and were also scattered among the axonal bundles.

The glaucomatous eyes either with POAG or NPG demonstrated backward bowing of the lamina cribrosa and axonal atrophy. The degree of the laminar bowing was comparable in the eyes with POAG or NPG. The degree of axonal atrophy was mild to moderate in the eyes with POAG and was especially noted in the postlaminar region. In the eyes with NPG, the axonal atrophy was moderate in most eyes and characterized with focal loss in the areas of cavernous degeneration. In 1 eye with NPG, severe axonal loss was noted through the optic disc cup, with axonal preservation in more peripheral areas. The postlaminar region of the optic nerve head in the eyes with POAG demonstrated mild disorganization of the pial septae without tissue destruction. These changes were uniformly consistent in all eyes with POAG. In contrast, the eyes from the patients with NPG exhibited varying stages of Schnabel cav-

Table 1. Clinical Data of Postmortem Glaucomatous Eyes*

Patient No./Age, y/Sex	Diagnosis	IOP Range, mm Hg	Vertical C/D Ratio	Visual Field Damage	Treatment
1/82/M	POAG	21-25	NA	NA	Topical β -blocker
2/78/M	POAG	12-28	NA	NA	Medical
3/74/F	POAG	16-28	0.7	Moderate	Medical
4/82/F	POAG	15-31	0.8	Moderate	Surgical + medical
5/84/F	NPG	11-14	0.95	Advanced	Topical β -blocker
6/84/F	NPG	11-14	0.95	Advanced	Topical β -blocker
7/68/F	NPG	14-18	0.8	Moderate	Topical β -blocker
8/82/F	NPG	13-17	0.8	Moderate	Topical β -blocker
9/82/F	NPG	13-17	0.8	Moderate	Topical β -blocker
10/74/F	NPG	13-18	0.8	Moderate	Topical β -blocker
11/74/F	NPG	12-19	0.9	Advanced	Topical β -blocker

*IOP indicates intraocular pressure; C/D, cup-disc; M, male; POAG, primary open-angle glaucoma; NA, not applicable; F, female; and NPG, normal-pressure glaucoma.

Table 2. Semiquantitative Evaluation of the Intensity of Immunostaining in Optic Nerve Head*

	MMP-1	MMP-2	MMP-3	TNF- α	TNF- α Receptor 1
Control (n = 4)	0.50	0.56	0.28	0.38	0.44
POAG (n = 4)	0.29	0.96	0.71	0.72	1.22
NPG (n = 7)	0.67	1.86	1.19	0.94	1.44

*MMP indicates matrix metalloproteinase; TNF- α , tumor necrosis factor α ; POAG, primary open-angle glaucoma; and NPG, normal-pressure glaucoma.

ernous degeneration, which was evident mainly at the lamina cribrosa and the postlaminar optic nerve. Axonal atrophy accompanied multifocal destruction of the cribriform laminar plates or pial septae within the cavernous degeneration areas seen in the eyes with NPG. In some eyes, the areas of degeneration coalesced to form large cavernous spaces. In the areas of preserved axons, the arrangement of laminar plates and pial septae remained intact and the distribution of glial cells remained unchanged.

Examinations of the optic nerve heads using immunohistochemistry revealed that the intensity of the immunostaining and the number of stained cells for MMPs, TNF- α , or TNF- α receptor 1 were greater in the glaucomatous optic nerve heads, particularly with NPG, when compared with age-matched controls. The immunolabeling seemed to be mainly in cells that resembled astrocytes by morphology. The semiquantitative scores of the immunostaining in samples examined are presented in **Table 2**. Since **Table 2** reflects the changes in the intensity of immunostaining but not the number of stained cells, photographs are also presented to optimally reflect changes that occur in glaucomatous optic nerve heads. Immunostaining patterns of optic nerve heads for MMPs, TNF- α , and TNF- α receptor 1 are also given below.

MMP-1

In normal eyes, faint immunostaining for MMP-1 was observed in the cytoplasm of a few glial cells, located mostly in the laminar and postlaminar regions of the optic nerve head. Faint staining was also noted around the axons or in the pial septae.

Although the intensity of immunostaining for MMP-1 was similar in all regions of the optic nerve head of glaucomatous eyes and in control eyes, the number of positively stained glial cells was greater in glaucomatous eyes, either with NPG or POAG. In addition, in glaucomatous eyes, immunostaining was positive around the axons in the postlaminar region (**Figure 1**).

MMP-2

In normal eyes, the prelaminar, laminar, and postlaminar regions of the optic nerve head exhibited faint immunostaining for MMP-2 in a few glial cells and around the axons.

However, both the intensity of the immunostaining and the number of stained glial cells were moderately increased in the prelaminar region of the optic nerve head, as well as along the laminar beams, in the glaucomatous eyes with either NPG or POAG. In the postlaminar region, positive immunostaining was seen around the axons and around the pial blood vessels in the glaucomatous eyes, which was more prominent in the eyes with NPG compared with those with POAG. The MMP-2 labeling in the eyes with NPG was also noted along the degenerating laminar plates and pial septae lining the cavernous spaces as well as within the astrocytes in these structures (**Figure 2**). In the postlaminar region of some eyes with NPG, areas of axonal preservation were seen adjacent to areas of severe axonal atrophy. In the areas of preserved axons, intracytoplasmic MMP immunolabeling was more intense than seen in the glial cells located in the areas of severe atrophy (**Figure 3, A**).

MMP-3

In the normal eyes, faint immunostaining for MMP-3 was observed in rare glial cells and around the axons in the all regions of the optic nerve head.

In the glaucomatous eyes, immunostaining of glial cells and their processes around the axons for MMP-3 demonstrated an increase in the all regions of the optic nerve head compared with controls. The increase in the labeling intensity was particularly evident in the glial cells and along the axons and pial septae in the eyes with NPG (**Figure 4**). Perivascular anti-MMP-3 immunostaining was also noted

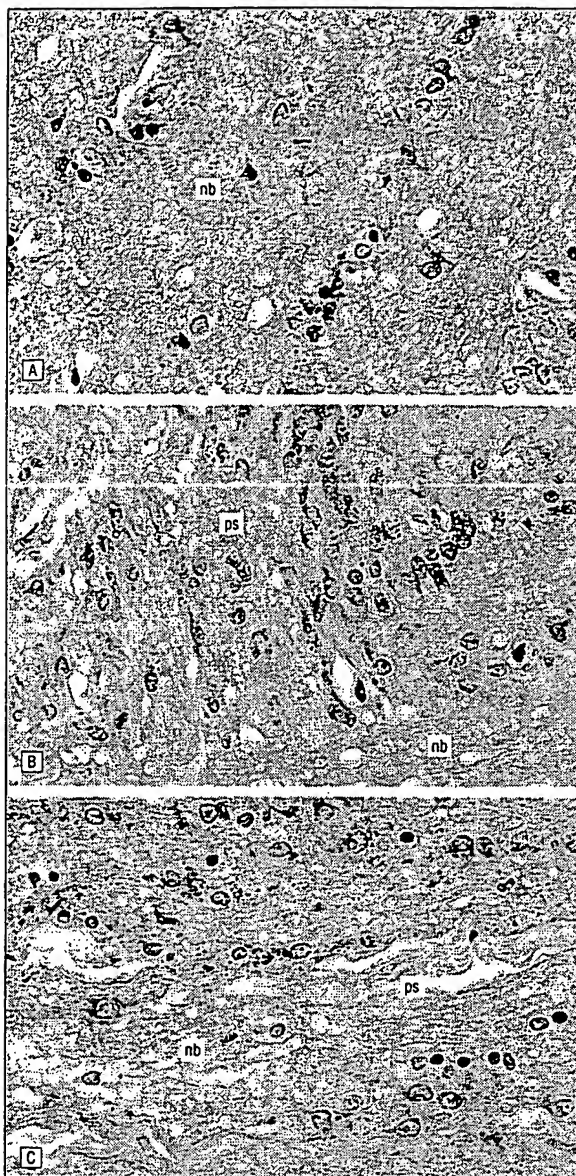


Figure 1. Immunoperoxidase staining for matrix metalloproteinase 1 (MMP-1) in the postlamina region of the human optic nerve head. There was faint immunostaining for MMP-1 in the cytoplasm of a few glial cells or their processes around the axons of the control optic nerve head (A). However, a greater number of glial cells demonstrated immunostaining for MMP-1 in the eyes with primary open-angle glaucoma (B) or normal-pressure glaucoma (C) (nb, nerve bundles; ps, pial septae) (chromogen, diaminobenzidine tetrahydrochloride, nuclear counterstain with Mayer hematoxylin, original magnification $\times 100$).

along the pial septae. In the eyes with NPG, glial cells located in the areas of preserved axons demonstrated more intensive immunostaining compared with the cells located in the areas of cavernous atrophy (Figure 3, B).

TNF- α

In control eyes, there was faint immunostaining for TNF- α and TNF- α receptor 1 in the processes of a few glial cells and around the nerve bundles and blood vessels of the optic nerve head.

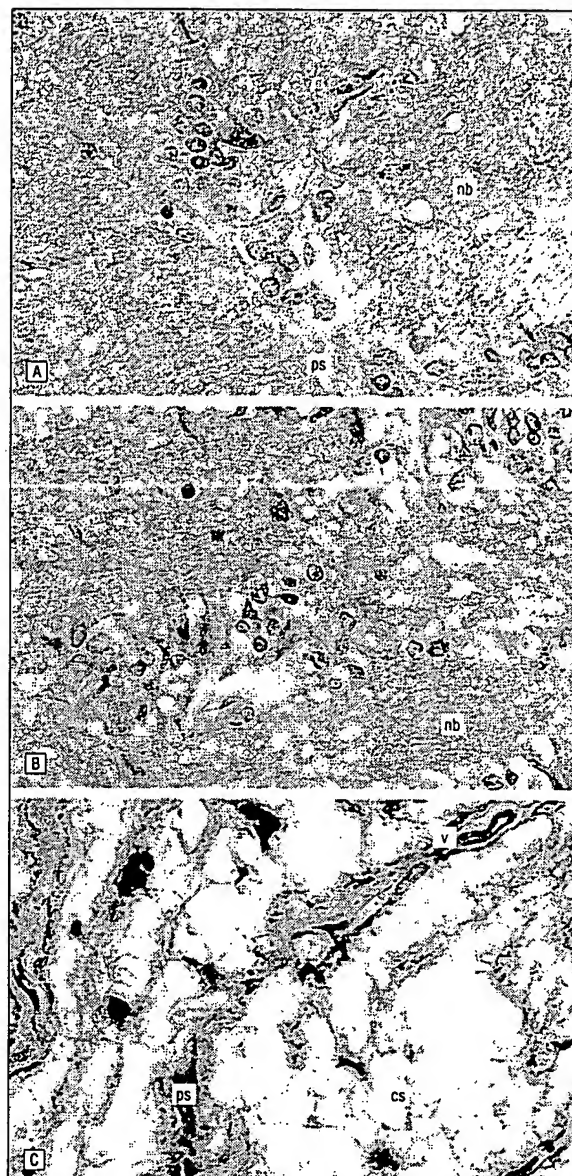


Figure 2. Immunoperoxidase staining for matrix metalloproteinase 2 (MMP-2) in the postlamina region of the human optic nerve head. There was faint immunostaining for MMP-2 in the cytoplasm of a few glial cells or their processes around the axons of the control optic nerve head (A). However, a greater number of glial cells demonstrated immunostaining for MMP-2 in the eyes with primary open-angle glaucoma (B) or normal-pressure glaucoma (C). Notice the intense immunostaining of glial cell processes in areas of cavernous atrophy and around pial blood vessels in the eye with normal-pressure glaucoma (nb, nerve bundles; ps, pial septae; cs, cavernous spaces; and v, vessels) (chromogen, diaminobenzidine tetrahydrochloride, nuclear counterstain with Mayer hematoxylin, original magnification $\times 100$).

In glaucomatous optic nerve heads, both the intensity of immunostaining and the number of stained cells for TNF- α or TNF- α receptor 1 were increased in all regions of the glaucomatous optic nerve head compared with controls. Immunostaining was positive in glial cells around the axons and vessels in the prelaminar and laminar regions of the optic nerve head in the glaucomatous eyes. In the postlamina region, the glial cells distrib-

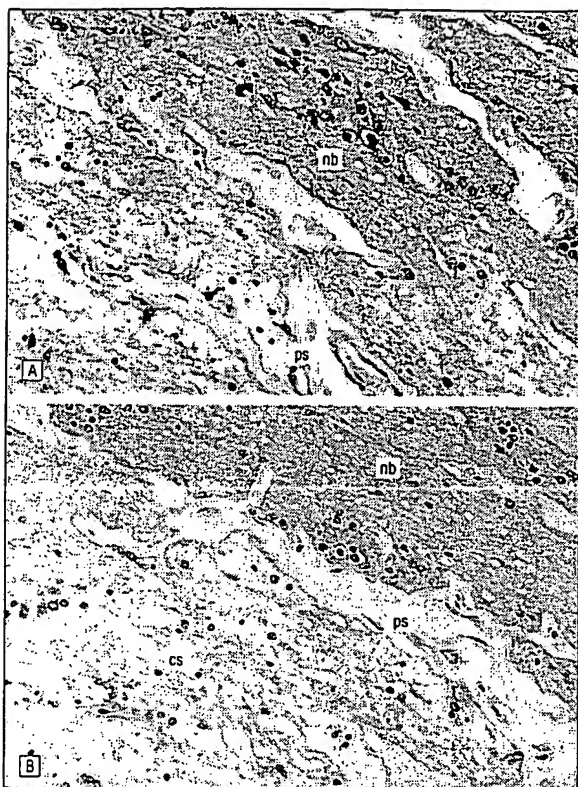


Figure 3. Immunoperoxidase staining for matrix metalloproteinases (MMPs) in the postlamellar region of the human optic nerve head. A junctional area between preserved (upper right corner) and severely damaged axons (lower left corner) in the optic nerve head of a patient with normal pressure glaucoma was seen. Immunostaining for MMP-2 (A) or MMP-3 (B) was more intense in the cytoplasm of astroglial cells in the areas of preserved axons compared with the areas of severe atrophy (nb, nerve bundles; ps, pial septae; and cs, cavernous spaces) (chromogen, diaminobenzidine tetrahydrochloride, nuclear counterstain with Mayer hematoxylin, original magnification $\times 100$).

uted along the pial septae and scattered among the nerve bundles exhibited immunostaining. Although immunostaining for TNF- α was mostly associated with glial cells, an increased immunostaining for TNF- α receptor 1 was also observed in the nerve bundles, which was prominent in the prelaminar region of the glaucomatous optic nerve heads (**Figures 5 and 6**).

COMMENT

The integrity and turnover of the extracellular matrix are influenced by many factors, including MMPs. The MMPs are a family of proteolytic enzymes secreted by glial cells, and are capable of degrading almost all components of the extracellular matrix. The MMPs have been divided into the following 3 broad families based on their domain structure and substrate specificity. (1) Interstitial collagenase (MMP-1) and neutrophil collagenase (MMP-8) belong to the collagenase family; their major substrates are fibrillar collagen types I, II, and III. (2) The enzymes MMP-2 and MMP-9 are members of the gelatinase family; their substrates include types IV and V collagen, and fibronectin, proteoglycans, and gelatin. (3) Members of the stromelysin family include MMP-3 (stromelysin, transin) and

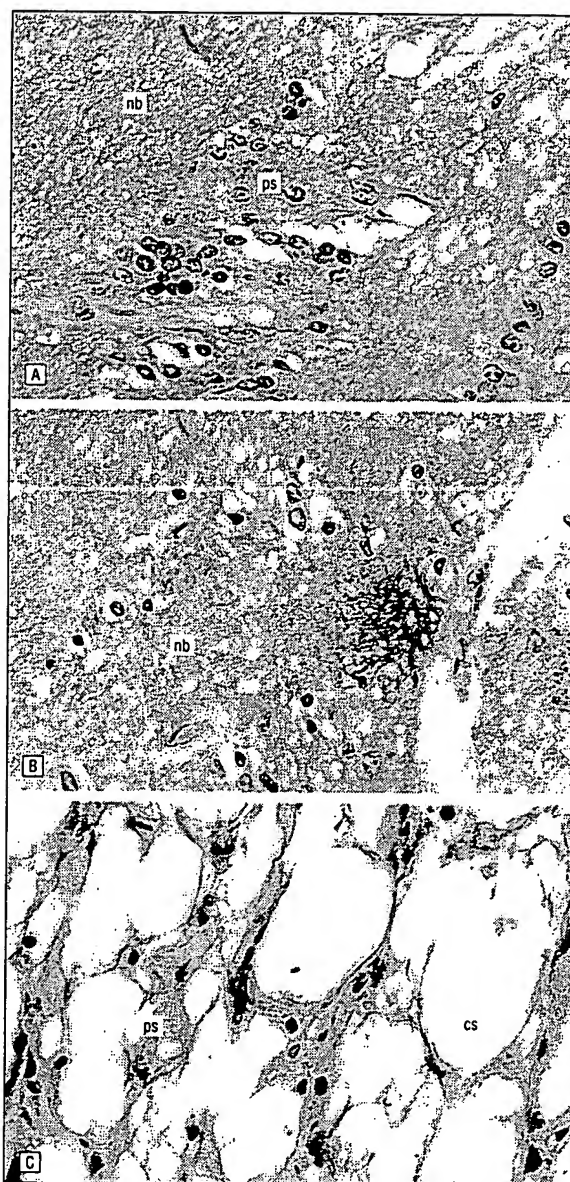


Figure 4. Immunoperoxidase staining for matrix metalloproteinase 3 (MMP-3) in the postlamellar region of the human optic nerve head. There was faint immunostaining in a few glial cells around the optic nerve axons of control eyes (A) and increased immunostaining in the processes of glial cells around the axons and in the pial septae of the eyes with primary open-angle glaucoma (B) or normal-pressure glaucoma in areas of cavernous atrophy (C) (nb, nerve bundles; ps, pial septae; cs, cavernous spaces; and v, vessel) (chromogen, diaminobenzidine tetrahydrochloride, nuclear counterstain with Mayer hematoxylin, original magnification $\times 100$).

MMP-7 (matrilysin); they act on a wide range of substrates, including proteoglycans, laminin, fibronectin, gelatin, and procollagen precursor peptides.^{15,19-23}

Although they are implicated in several diseases of the central nervous system,¹¹⁻¹³ little is known about the role of MMPs in either normal or glaucomatous human optic nerves. The localization of MMP-3 and MMP-2 and tissue inhibitor of metalloproteinases (TIMP-1) have been shown to be present in the normal primate optic nerve head and retina.²⁴ In addition, increased gelatinase ac-

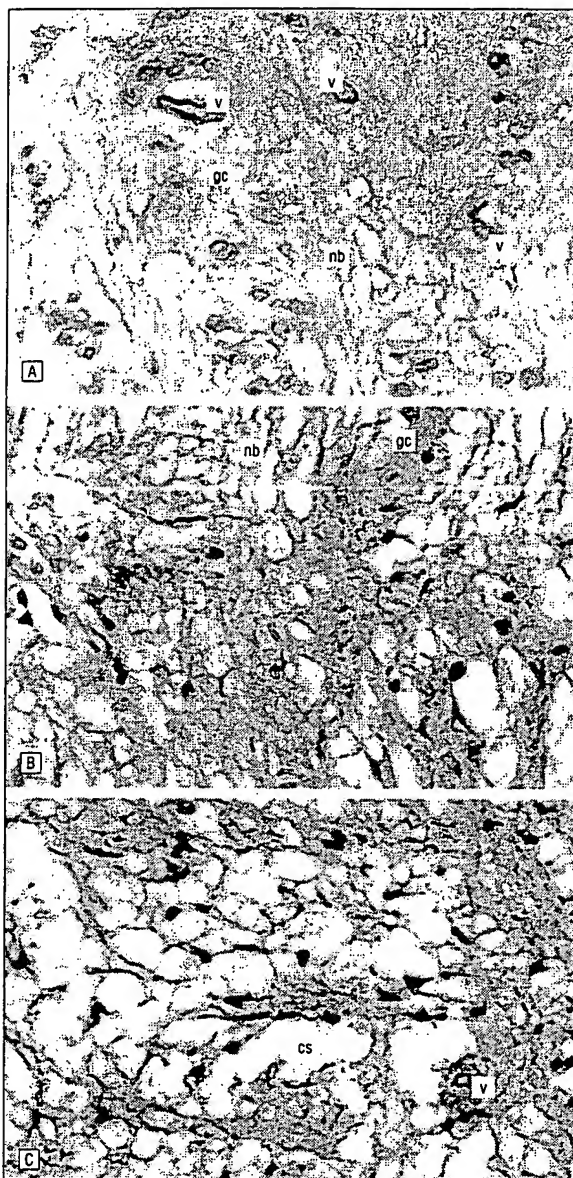


Figure 5. Immunoperoxidase staining for tumor necrosis factor α (TNF- α) in the human optic nerve head. There was faint immunostaining in the processes of a few glial cells around the nerve bundles and blood vessels (v) in the prelaminar region of the control optic nerve head (A). However, the intensity of the immunostaining and the number of stained glial cells were greater in the optic nerve heads from patients with primary open-angle glaucoma (B) or normal-pressure glaucoma (C). There was intense immunostaining of glial cell processes in areas of cavernous atrophy in the eye with normal-pressure glaucoma (gc, glial column; nb, nerve bundles; and cs, cavernous spaces) (chromagen, diaminobenzidine tetrahydrochloride, nuclear counterstain with Mayer hematoxylin, original magnification $\times 100$).

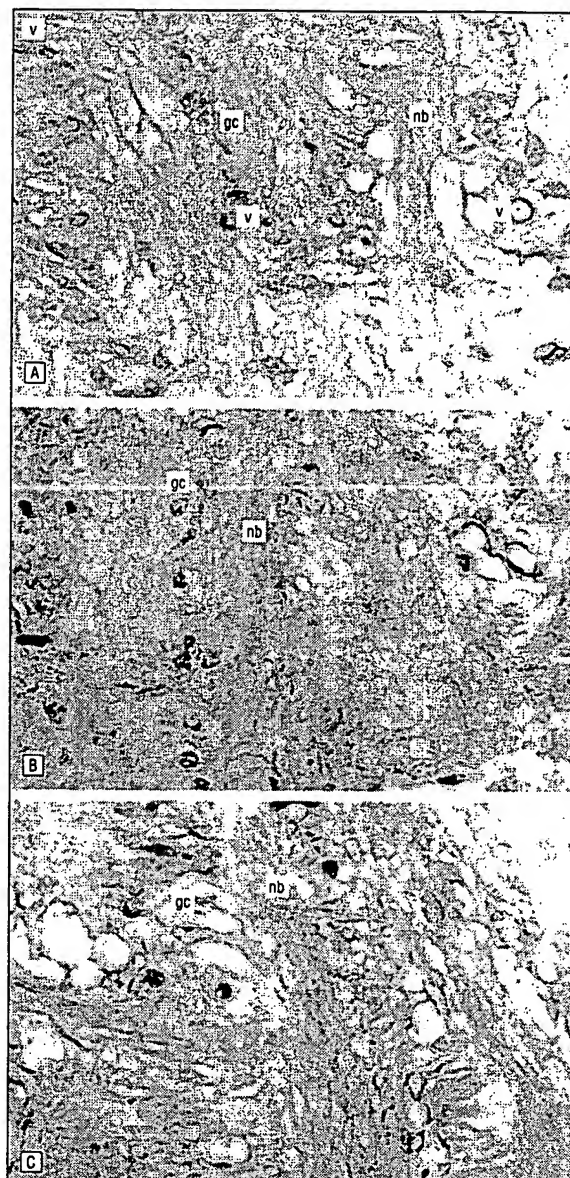


Figure 6. Immunoperoxidase staining for tumor necrosis factor α (TNF- α) receptor 1 in the human optic nerve head. Faint immunostaining of the prelaminar region of the optic nerve head was noted for TNF- α receptor 1 in the control optic nerve head (A). Immunostaining was mostly perivascular (v). The intensity of the immunostaining and the number of stained glial cells were greater in optic nerve heads from patients with primary open angle glaucoma (B) or normal pressure glaucoma (C). Nerve bundles in the prelaminar region also exhibited some immunostaining (gc, glial column; nb, nerve bundles) (chromagen, diaminobenzidine tetrahydrochloride, nuclear counterstain with Mayer hematoxylin, original magnification $\times 100$).

tivity has been found in glaucomatous monkey eyes.^{25,26} Our observation of the mild MMP immunolabeling of the glial cells in normal optic nerve heads and increased immunolabeling of MMPs in glaucomatous eyes is consistent with these limited studies.

Our observations revealed that the intensity of immunostaining for MMPs, TNF- α , and TNF- α receptor 1 was greater in glaucomatous optic nerve heads compared with controls. In addition, differential immunostaining pat-

terns for these proteins were noted in the prelaminar, laminar, and postlaminar regions of the optic nerve head. Some of these differential patterns included the most prominent labeling of MMPs in the postlaminar region and the most prominent labeling of TNF- α and TNF- α receptor 1 in the prelaminar region of the glaucomatous optic nerve heads. One possible explanation of these findings may be based on the recently described regional and functional heterogeneity of glial cells in the optic nerve head. For ex-

ample, the size and the density of type 1B astrocytes in the prelaminar and laminar regions, and the type 1A astrocytes in the postlaminar region, are greater in glaucomatous eyes than in normal tissue.²⁷⁻²⁹

Increased immunostaining of MMPs was noted in the cytoplasm of astroglial cells and their processes as well as in the extracellular matrix of optic nerve head in the eyes with POAG or NPG. The distribution of increased immunostaining for MMPs in the different regions of optic nerve head was comparable in the eyes with POAG or NPG. However, the intensity of immunostaining for MMPs, especially for MMP-2, was greater in the eyes with NPG compared with the eyes with POAG. In the eyes with NPG, immunostaining along the pial septae was moderately increased in the region of cavernous degeneration.

Cells secrete MMPs in an inactive form and the proenzyme can be activated in the extracellular space by various molecules. The antibodies used to recognize MMPs in this study identify both MMP precursors and the proteolytically processed active forms. Therefore, immunohistochemistry cannot distinguish the functional state in which the MMPs are present within the tissue. The abundance of immunoreactivity in the astrocytes suggests the presence of a large pool of intracellular MMPs that might function, under normal conditions, at relatively low levels in the extracellular space. Such pools could possibly be rapidly activated to act on substrates in the extracellular matrix under pathologic conditions.²³

The generalized increase in the expression of MMPs in the glaucomatous optic nerve head may have various consequences. Since MMPs are responsible for the degradation of the extracellular matrix components, their increased expression in the glaucomatous optic nerve head may represent a physiological response to counteract the increased extracellular matrix deposition that occurs in glaucomatous optic nerve head.³⁰ This may explain the absence of glial scar tissue in glaucomatous optic nerves despite astroglial activation. It is tempting to speculate that tissue degeneration resulting from increased MMP activity may in part account for the excavated appearance of optic disc cupping that accompanies glaucomatous optic neuropathy, regardless of other factors such as intraocular pressure.

Matrix metalloproteinases have been proposed to play a role in axonal growth by preventing scar tissue formation *in vivo*,^{31,32} which is thought to be a barrier to trophic substances necessary for neuronal regeneration.³³ Therefore, our observation of prominent immunostaining for MMPs in the areas of preserved axons may signify that activated glial cells increase secretion of MMPs for the dual purposes of preventing scar tissue formation while simultaneously promoting neuronal growth.

The pial septae of the normal optic nerve contains collagen types III and IV and fibronectin mainly around the blood vessels.³⁴ These are the major substrates of MMP-2 and MMP-3. The increased immunostaining of MMP-2 and MMP-3 in the astrocytes and along the pial septae in the glaucomatous optic nerve head suggests that these MMPs may play a role in the disruption of pial septa seen in the areas of cavernous degeneration.

In addition, we observed increased expression of MMP-2 in the astrocytic processes enveloping blood vessels in the glaucomatous optic nerve head, particularly

in the eyes with NPG. Since MMP-2 causes a thinning of the basal lamina and an increase in the capillary permeability,³⁵ it seems possible that increased expression of MMPs in the perivascular area may influence the blood-brain barrier in this area.

Another finding we observed was increased immunostaining of TNF- α and TNF- α receptor 1 in the glaucomatous optic nerve heads either with POAG or NPG. Tumor necrosis factor α is a potent immunomediator and proinflammatory cytokine that is rapidly up-regulated in the brain after injury.^{36,37} It is also known as an inducer of apoptotic cell death via TNF- α receptor 1 occupancy.³⁸ Tumor necrosis factor α has been implicated in the pathogenesis of several diseases of the nervous system, such as multiple sclerosis and autoimmune encephalomyelitis; it has also been thought to account for axonal degeneration and glial changes observed in the optic nerves of patients with acquired immunodeficiency syndrome.³⁹ Although our studies demonstrated that the TNF- α immunostaining was mostly positive in the glial cells of the optic nerve head, TNF- α receptor 1 immunostaining was more prominently positive in nerve bundles located in the prelaminar section of the optic nerve head, which was increased in the glaucomatous eyes. This observation suggests that neuronal tissue is an important target for the effects of TNF- α . Our findings that the expression of TNF- α and MMPs are both increased in the glaucomatous optic nerve head is not surprising, since it is well known that there are interactions between TNF- α and MMPs for the regulation of their secretion and function.¹⁴⁻¹⁸ Therefore, increased expression of TNF- α in the glaucomatous optic nerve head suggests that this cytokine may play a role in tissue remodeling as a part of the astroglial activation process and/or may participate in tissue injury.

In addition to its potential to directly activate the cell death cascade in retinal ganglion cells and to facilitate remodeling of the optic nerve head in glaucoma, TNF- α may also contribute to the pathogenesis of glaucomatous neuropathy, as it is a potent stimulator of nitric oxide synthesis.⁴⁰⁻⁴² Recent evidence suggests that up-regulation of nitric oxide synthase occurs in human and experimental glaucomatous eyes.⁴³ Furthermore, pharmacological inhibition of nitric oxide synthase-2 was shown to decrease ganglion cell death in an experimental animal model of glaucoma.⁴⁴ Therefore, blockade, amelioration, or attenuation of retinal or optic nerve head TNF- α may have therapeutic potential in treating patients with glaucoma. Such compounds could effectively inhibit, reduce, or prevent nitric oxide synthase-related ganglion cell death, which may be an important causal factor in glaucoma.

CONCLUSIONS

Increased expression of MMPs, TNF- α , and TNF- α receptor 1 may be collective components of the astroglial activation process that occurs in the glaucomatous optic nerve head. They may serve to prevent scar tissue formation and thus facilitate neuronal viability and repair. However, their increased expression may also have a role in the degenerative process of glaucomatous optic neuropathy as a result of facilitating formation of cavernous spaces, cupping, and the progression of neuronal damage.

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Cytokine Involvement in Cancer Anorexia/Cachexia: Role of Megestrol Acetate and Medroxyprogesterone Acetate on Cytokine Downregulation and Improvement of Clinical Symptoms

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ABSTRACT: The characteristic clinical picture of anorexia, tissue wasting, loss of body weight accompanied by a decrease in muscle mass and adipose tissue, and poor performance status that often precedes death has been named the cancer-related anorexia/cachexia syndrome (CACS). Chronic administration of pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF) either alone or in combination, is capable of reproducing the different features of CACS. High serum levels of these cytokines have been found in cancer patients, which seem to correlate with progression of the tumor. This paper describes a series of experimental and clinical studies demonstrating that: (1) high serum levels of some cytokines, including IL-1, IL-6, and TNF, are present in advanced-stage cancer patients, particularly those with CACS; (2) megestrol acetate (MA) has a beneficial therapeutic effect on CACS symptoms, such as appetite, body weight, and quality-of-life; (3) MA downregulates the synthesis and release of cytokines and relieves the symptoms of CACS; (4) cytokines play a key role in the onset of CACS; (5) medroxyprogesterone acetate (MPA) reduces the *in vitro* production of cytokines and serotonin (5-hydroxytryptamine, 5-HT) by peripheral blood mononuclear cells (PBMC) of cancer patients; and (6) MA and MPA reduce the cisplatin-induced 5-HT release *in vitro* from PBMC of cancer patients. Based on these results, a clinical study incorporating MA/MPA in combination with chemotherapy or chemo-immunotherapy may be warranted.

KEY WORDS: cancer anorexia/cachexia syndrome, cytokines, megestrol acetate, medroxyprogesterone acetate.

I. INTRODUCTION

The characteristic clinical picture of anorexia, tissue wasting, the loss of body weight accompanied by a decrease in muscular mass and adipose tissue and the poor performance status preceding death, so often found in advanced stage cancer patients, was named cancer-related anorexia/cachexia syndrome (CACS) (Heber et al., 1986; Nelson and Walsh, 1991; Bruera, 1992; Brennan, 1997). It is the most common single cause of death documented in these patients, affecting about half of all cancer patients (Warren, 1932).

Anorexia is one of the main features of the cachectic syndrome: although its etiopathogenesis is most certainly multifactorial and not yet well understood, it seems at least in part attributable to intermediary metabolites that accumulate along an abnormal metabolic pathway in cancer patients (lactate, ketones, oligonucleotides) or other substances released by the tumor itself or by the host in response to the tumor (Bruera, 1992). However, anorexia by itself cannot explain the complex organic alterations seen in CACS: indeed, nutritional supplementation alone is not able to effectively reverse the process of cachexia. An

increased resting energy expenditure may contribute to weight loss in cancer patients and may explain the increased oxidation of fat. Futile energy-consuming cycles, such as the Cori cycle, may contribute to the increased energy demand.

Unlike starvation, weight loss in cancer arises equally from loss of muscle and fat, and the process is characterized by an increased catabolism of skeletal muscle and a decrease in protein synthesis (Tisdale, 1997a). In addition to the reduced food intake, important biochemical/metabolic abnormalities and changes in energy metabolism have been observed, which may account for the cachectic syndrome. The most important carbohydrate abnormalities are increased glucose synthesis, insulin resistance, decreased glucose tolerance and turnover, increased gluconeogenesis and increased Cori cycle activity. Increased protein turnover, decreased muscle protein synthesis, increased muscle catabolism and increased liver and tumor protein synthesis are the main pathologic changes of protein metabolism. The main abnormalities found in fat metabolism are enhanced lipid mobilization, decreased lipogenesis, decreased lipoprotein lipase activity, elevated triglycerides and decreased high-density lipoproteins, increased venous glycerol and decreased glycerol clearance from the plasma (Heber et al., 1986; Devereaux et al., 1984; Vlassara et al., 1986). Catabolic factors capable of direct breakdown of muscle and adipose tissue appear to be secreted by cachexia-inducing human tumors and may play an active role in the process of tissue degeneration (Tisdale, 1997a).

CACS may result from circulating factors produced by the host or by the tumor itself. A number of cytokines, host-derived protein molecules released by lymphocytes and/or monocytemacrophages, including Interleukin- (IL-)1 and IL-6, Tumor Necrosis Factor (TNF) α and Interferon (IFN) γ , have been proposed as mediators of the cachectic process; however, the results of numerous clinical and laboratory studies suggest that the action of cytokines alone is unable to explain the complex mechanism of CACS (McNamara et al., 1992; Espat et al., 1992; Noguchi et al., 1996; Tisdale, 1997b).

High serum levels of IL-1, IL-6, and TNF α have been found in cancer patients and the levels of these cytokines seem to correlate with the

progression of the tumor (Moldawer et al., 1987, 1988, 1992; Strassmann et al., 1992). The chronic administration of these factors in man, either alone or in combination, is capable of reproducing the different features of CACS (Moldawer et al., 1988; Busbridge, 1989; Gelin et al., 1991; McLaughlin et al., 1992; Strassmann et al., 1992). More direct evidence of a cytokine involvement in CACS is provided by the observations that cachexia in experimental animal models (Sherry et al., 1991; Noguchi et al., 1996; Matthys and Billiau, 1997) can be relieved by administration of specific antagonists of cytokines: these studies revealed that cachexia can rarely be attributed to any one single or specific cytokine but rather to a set of cytokines that work in concert in cachexia. The same cytokines seem to play central roles in the cachexia-related inflammation as well as in the acute phase response (Moldawer and Copeland, 1997).

II. CLINICAL EXPERIENCE WITH MEDROXYPROGESTERONE ACETATE AND MEGESTROL ACETATE

Two of our studies contributed to support the central role of cytokines in CACS. The first was carried out in nine cancer patients with head and neck carcinoma (HNC) (Mantovani et al., 1995) and the second in a population of 10 patients with cancer of different sites (Mantovani et al., 1997). Both demonstrated abnormally high serum levels of IL-1 β , IL-6, TNF α , and serotonin (5-HT), and a high production of the same cytokines by phytohemagglutinin-(PHA-) stimulated cultured peripheral blood lymphocytes (PBMC) from cancer patients when compared with normal subjects.

The first study was carried out to evaluate the effect of megestrol acetate (MA) in CACS, that is, the ability of MA to induce an increased appetite and body weight in HNC patients in an advanced (III-IV) stage of the disease treated with cisplatin-based neoadjuvant chemotherapy. The clinical evaluation was paralleled by the assessment of serum levels of the cytokines IL-1 α and β , IL-2, IL-6, TNF α , and the soluble receptor for IL-2 (sIL-2R) in all patients before and after MA treatment. The same cytokines and sIL-2R were also measured in the culture medium of PHA-

stimulated PBMC of the same patients before and after MA treatment. The choice of MA came from consideration that progestins are among the drugs known to be the most effective in controlling CACS symptoms by stimulating appetite and increasing food intake and body weight (Heber et al., 1986). In particular, MA (Tchekmedyan et al., 1986, 1987, 1992; Aisner et al., 1988; Bruera et al., 1990; Loprinzi et al., 1990) and, to a lesser extent, medroxyprogesterone acetate (MPA) (Downer et al., 1993; Simons et al., 1996), both active oral synthetic derivatives of the natural steroid progesterone have been used extensively for increasing caloric intake and body weight mostly due to an increased deposition of fat (Reitmeier et al., 1990; Loprinzi et al., 1993), and were shown to be well tolerated. Subsequent trials were designed to find the optimal dose of MA (Schmoll et al., 1991; Loprinzi et al., 1994; Gebbia et al., 1996). The effect of MA on CACS induced by several different types of cancer had been studied previously, but data were lacking on the effect of MA on the appetite and nutritional state of patients with advanced HNC undergoing chemotherapy or chemotherapy combined with radiation therapy. Among neoplastic patients, those affected by primary HNC represent a group in which the loss of appetite, the difficult alimentation due to anatomical factors, weight loss, and the more or less serious state of malnutrition (often already present before diagnosis of the neoplasia) associated with the bad habits of these patients (they are nearly always heavy smokers and/or drinkers), eventually made worse by the effects of antineoplastic chemotherapy, induce a severe CACS in a relatively early stage of the neoplasia, so much so that it represents a paradigmatic pattern of this type of cancer. Eleven male patients (mean age 57.8 years, range 43 to 69, PS K 90 to 100, weight decrease > 10% of the ideal or customary body weight) were enrolled in our study. Ten patients were treated with MA during neoadjuvant chemotherapy, while one patient was treated with MA during definitive locoregional radiation therapy administered at the end of primary chemotherapy. The neoadjuvant chemotherapy consisted of either the Al-Sarraf's regimen: cisplatin 100 mg/m² i.v. on day 1 plus fluorouracil (5-FU) 1000 mg/m²/day i.v. continuous infusion (c.i.) on days 1 to 5 repeated every 3

weeks or the same regimen plus vinorelbine (20 mg/m² i.v. days 2 and 8). The clinical parameters evaluated were clinical response to chemotherapy after three cycles, body weight, appetite (using a visual analogue scale calibrated from 0 to 10), PS K, quality of life (Spitzer's Quality of Life Index) (Spitzer et al., 1981) before and after MA treatment. The immunological parameters studied were serum levels of IL-1 α and β , IL-2, IL-6, TNF α , and sIL-2R in cancer patients before and after MA treatment and compared with those of normal individuals and the production in cultures of the same cytokines by PHA-stimulated PBMC of patients before and after MA treatment. MA (MEGESTIL[®], Boehringer-Mannheim, Milan, Italy, tablets of 160 mg) was administered at a dose of 320 mg/day during the interval between cycles of chemotherapy, starting from the third day after the end of cycle until the day before the next cycle (days 8 to 21) for a total of three consecutive cycles. During the cycles the dose ranged from 320 to 160 mg/day according to clinical response. Of the 11 enrolled patients, 9 (81.8%) were evaluable (2 patients were not evaluable due to major protocol violations: drug intake < 90% of that programmed). The clinical parameters before and after MA treatment are reported in Table 1. Except for PS K, all the parameters showed an improvement following treatment with MA. In particular, average body weight increased by 6.3 kg (13.2%), appetite by a score of 2.4 (38.6%), and the Spitzer's QLI by a score of 2.4 (36.2%). The serum levels of cytokines studied were significantly higher in patients before MA treatment than in normal subjects. Levels of all cytokines decreased in patients after MA treatment and decreases were statistically significant particularly for IL-1 β . The production in culture of the same cytokines by PBMC from patients before MA treatment was not significantly different from that of normal subjects, except for IL-6. As for cytokine production by patient PBMC after MA treatment, IL-6 decreased significantly. Our study was, to our knowledge, the first published that correlated the clinical response to MA with serum levels and cultured PBMC production of the cytokines known to be involved in CACS. Despite the small number of patients included, we believe that our study was noteworthy because of the very homogeneous

TABLE 1
Evaluation of Clinical Parameters in Patients Treated with Chemotherapy and MA

		Treatment with MA		Mean Increase	%	p
		Before	After			
Weight (kg)	Mean \pm SD	47.3 \pm 7.8	53.6 \pm 12.5	+6.3	13.2	< 0.0
	Range	34-83	28.5-70			
Appetite (score)	Mean \pm SD	6.3 \pm 2.0	8.7 \pm 1.3	+2.4	38.6	0.00
	Range	2-9	6-10			
PSK (score)	Mean \pm SD	98.7 \pm 5.0	94.4 \pm 16.7	-2.3	-2.3	NS
	Range	90-100	50-100			
Spitzer's QLI (score)	Mean \pm SD	6.4 \pm 1.3	8.8 \pm 1.7	+2.4	36.2	0.00
	Range	5-9	6-10			

Note: Abbreviations: MA, megestrol acetate; PSK, Karnofsky performance status; QLI, quality of life index; SD, standard deviation; NS, nonsignificant. Statistical analysis was performed with *t*-test for paired data.

patient population studied and the very strong correlation between the type of tumor and CACS. Our study strongly supported the hypothesis that the beneficial therapeutic effects of MA in CACS may at least in part be due to its ability to downregulate the synthesis and release of the key cytokines involved, blocking the cascade of events responsible for CACS.

Our subsequent study (Mantovani et al., 1997) addressed the question whether the other more widely used synthetic progestagen, MPA (PROVERA®, Pharmacia & Upjohn, Milan, Italy), at pharmacological doses *in vitro* (0.1, 0.2, and 0.4 μ g/ml), was able to influence the production and/or release in culture of IL-1 β , IL-2, IL-6, TNF α , and 5-HT by PHA-stimulated PBMC of advanced

stage cancer patients. Ten patients (mean age 62.5 years, range 54 to 78; M/F 8/2; 6 HNC, 2 colon ca, 1 non-small cell lung ca—NSCLC—, 1 ovarian ca; PS ECOG 0-1: 7 patients, 3-4: 3 patients) were studied. The levels of cytokine production were significantly higher in cancer patients than in controls. The addition into culture of MPA at 0.2 μ g/ml significantly reduced the production of IL-1 β , IL-6, TNF α , and 5-HT but not IL-2 (Table 2). The concentration of MPA we selected (0.2 μ g/ml) as the most effective for our study may be reached *in vivo* following very high-dose MPA administration (1500 to 2000 mg/day orally), such as that used for endocrine therapy of hormone-related cancers and, to a lesser extent, that used as supportive care for anorexia/cachexia in cancer

TABLE 2A
Cytokine and 5-HT Production from PHA-Stimulated PBMC of Cancer Patients and Normal Subjects

Cytokines	Patient PBMC	p	Normal PBMC
IL-1 β	3531 \pm 317	< 0.05	2470 \pm 294
IL-2	2194 \pm 228	< 0.001	437 \pm 54
IL-6	6528 \pm 380	< 0.001	2160 \pm 38
TNF α	4489 \pm 327	< 0.001	945 \pm 52
5-HT	1188 \pm 183	0.001	75 \pm 5

Note: Results are expressed as mean pg/mL plus minus standard error (or as nM plus minus standard error for 5-HT).

TABLE 2B

Cytokine and 5-HT Production from Cancer Patients PBMC Stimulated With PHA \pm 0.2 μ g/ml Medroxyprogesterone Acetate

Cytokines	PHA	<i>p</i>	PHA + MPA 0.2 μ g/ml
IL-1 β	3531 \pm 317	< 0.05	2464 \pm 337
IL-2	2194 \pm 228	NS	1947 \pm 107
IL-6	6528 \pm 360	0.01	5123 \pm 334
TNF α	4469 \pm 327	< 0.05	3142 \pm 354
5-HT	1188 \pm 183	< 0.05	690 \pm 107

Note: Results are expressed as mean pg/ml plus minus standard error (or as nM plus minus standard error for 5-HT). Abbreviations: MPA, medroxyprogesterone acetate; NS, nonsignificant.

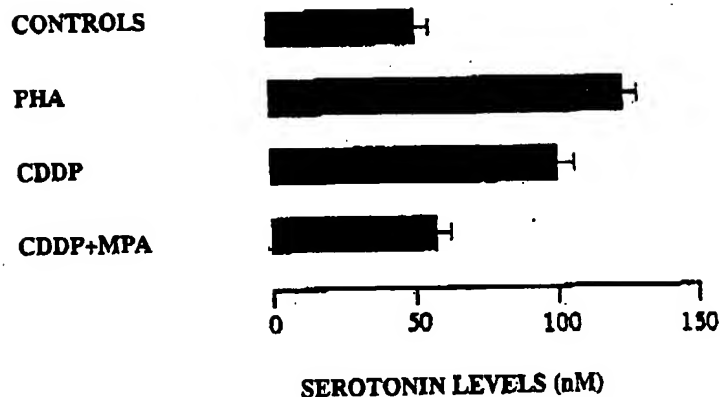
patients. The study showed that the production in culture of IL-1 β , IL-6, TNF α , and 5-HT by PHA-stimulated PBMC of cancer patients is significantly reduced in the presence of MPA. A further interesting finding of our study was that MPA is able to inhibit 5-HT production and release by PBMC. Recently, it has been demonstrated that 5-HT, as well as cytokines, plays an important role in upregulating T-lymphocyte function *in vitro* and cell-mediated immunity *in vivo* (Aune et al., 1994). Therefore, it can be hypothesized that, along with the cytokines, high amounts of 5-HT may be produced in advanced stage cancer patients as a consequence of the chronic activation of the immune system by specific (tumor antigens?) or nonspecific stimuli. 5-HT is the main mediator of nausea and vomiting, which are common and distressing symptoms often associated with neoplasia itself and furthermore worsened by most antineoplastic treatments, such as cisplatin chemotherapy (Mantovani et al., 1996a); moreover, it is well known that 5-HT plays a key role in the onset of CACS (Storey, 1994). The MPA-mediated inhibition of 5-HT production found in our study may explain the previous reports that short-term administration of MPA can be effective on emesis induced by different combination chemotherapies (Suzuki, 1991). The relationships between 5-HT and cytokines involved in the pathogenesis of CACS still need to be clarified. Noteworthy, our results show that MPA is unable to interfere with both the activity

of IL-2 on lymphocytes and with IL-2R expression by the same cells.

A further step of our investigation was to verify whether cisplatin can induce *in vitro* 5-HT release from human PBMC: we showed that cisplatin did induce 5-HT release from PBMC dose dependently. Our results highlighted a new mechanism through which cisplatin could induce emesis (Mantovani et al., 1996b).

Furthermore, we evaluated the ability of either MPA or MA to reduce cisplatin-induced *in vitro* 5-HT release from PBMC of cancer patients (Mantovani et al., 1998a). Sixteen patients with cancer of different sites, all in advanced stage of disease, were studied (10 studied for MPA and 6 for MA). The levels of 5-HT in culture supernatant fluids of patient PBMC stimulated with cisplatin were in the same range as those of PHA-stimulated PBMC and were higher than unstimulated PBMC (100 nM vs. 51 for MPA study and 123 nM vs. 64 for MA study). The addition to cultures of MPA 0.2 μ g or MA 0.38 μ g was able to significantly reduce the cisplatin-induced production of 5-HT (62 nM for MPA study and 46 nM for MA study) (Figures 1 and 2). This finding, to our knowledge, has not been reported previously. The concentrations of MPA and MA used in cultures were in the same range as those raised in plasma following the clinical administration of daily doses of 1000 to 2000 mg of MPA and 320 to 960 mg of MA.

The above-mentioned experimental and clinical results prompted us to perform a phase I study



Results are expressed as mean \pm SEM

CONTROLS vs PHA: $p < 0.001$

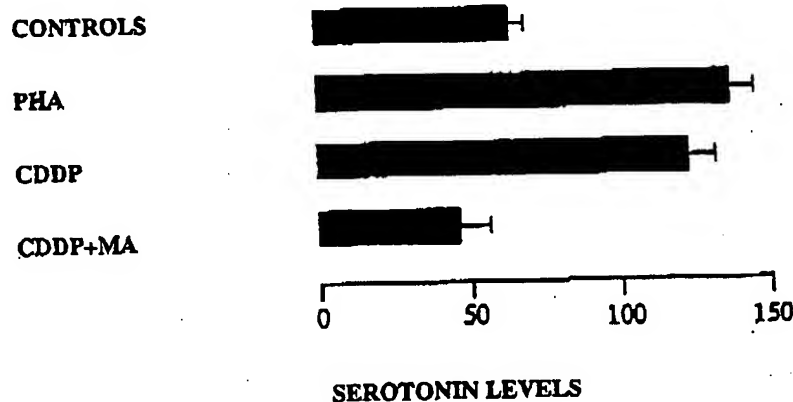
CONTROLS vs CDDP: $p < 0.001$

CDDP vs CDDP+MPA: $p < 0.001$

FIGURE 1. Serotonin levels in culture supernatants of PBMC in the absence (controls) or presence of PHA 0.5 μ g or CDDP 0.01 μ g, or CDDP plus MPA 0.2 μ g.

incorporating MPA into a combination of chemotherapeutic agents in patients with advanced (stage IIIB-IV) inoperable NSCLC (Mantovani et al., 1998b). The endpoints of the study were clinical response, and, for patients having symptoms, the relief of CACS symptoms, such as increase of appetite and body weight and PS improvement.

The serum levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, TNF α , and sIL-2R were assessed before any treatment, after treatment with MPA alone (1 week) and after six cycles of therapy. The weekly schedule was a combination of cisplatin i.v. day 1, epirubicin i.v. day 1, MPA (PROVERA®) 1 g/day p.o. days 1 to 7 and rIL-2 (PROLEUKIN®),



Results are expressed as mean \pm SEM

CONTROLS vs PHA: $p < 0.001$

CONTROLS vs CDDP: $p < 0.001$

CDDP vs CDDP+MA: $p < 0.001$

FIGURE 2. Serotonin levels in culture supernatants of PBMC in the absence (controls) or presence of PHA 0.5 μ g or CDDP 0.01 μ g, or CDDP plus MA 0.38 μ g.

Chiron, Milan, Italy) 1.8 MIU days 3 to 7 s.c. plus G-CSF support (300 µg days 2 to 7 s.c.). Administration of MPA started 1 week before the first cycle. Dose escalation was: cisplatin 30 mg/m²/w + epirubicin 25 mg/m²/w (1st level: 2 patients), cisplatin 30 mg/m²/w + epirubicin 33 mg/m²/w (2nd level: 2 patients), cisplatin 40 mg/m²/w + epirubicin 33 mg/m²/w (3rd level: 5 patients), cisplatin 40 mg/m²/w + epirubicin 40 mg/m²/w (4th level: 4 patients). After 6 weekly cycles, an evaluation of clinical response and toxicity was done. If become operable, patients underwent surgery; if not, three further cycles were administered. After nine cycles, if operable, patients underwent surgery, if not, three further cycles were administered. Inclusion criteria were: histologically confirmed primary lung cancer without brain metastases, ECOG PS 0-1, normal cardiac function (LVEF ≥ 60%, normal Echo). All patients gave written informed consent. From March to October 1997, 13 patients (M/F 11/2, mean age 59 years, range 42 to 68) were enrolled: all 13 were evaluable for toxicity and 11 of them for response. Eleven patients had NSCLC (2 patients had stage IV disease, 7 patients stage IIIB, 1 patient stage IIIA and 1 patient stage II, the last 2 patients were evaluated only for toxicity); 2 patients had SCLC (1 ED and 1 LD). As for the first endpoint, that is, clinical response after six cycles, 7 PR (63.63%), 3 SD (27.28%), and 1 PD (9.09%) out of 11 patients were found with an ORR of 63.63%. Only hematological toxicity was observed. Maximal toxicity per patient was as follows: 1 grade I (leukopenia) and 1 grade II (anemia) at 1st level (23 cycles evaluated); 1 grade II and 1 grade III (both anemia) at 2nd level (20 cycles evaluated); 1 grade I (anemia), 1 grade II (anemia and leukopenia), and 2 grade III (1 anemia and 1 leukopenia) at 3rd level (31 cycles evaluated); 1 grade I (anemia), 1 grade II (anemia), and 2 grade III (1 anemia and 1 leukopenia) at 4th level (29 cycles evaluated). One patient died while in PR for nontoxicity-related causes. As for the second endpoint, the only data available up to now are the serum levels of IL-1β, IL-2, IL-6, TNFα, and sIL-2R of patients before treatment: they were significantly higher than those of normal subjects. The comparison between serum levels of cytokines before and after treatment is under evaluation. The study is still ongoing.

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REVIEW

Biology of Cachexia

Michael J. Tisdale*

About half of all cancer patients show a syndrome of cachexia, characterized by loss of adipose tissue and skeletal muscle mass. Such patients have a decreased survival time, compared with the survival time among patients without weight loss, and loss of total body protein leads to substantial impairment of respiratory muscle function. These changes cannot be fully explained by the accompanying anorexia, and nutritional supplementation alone is unable to reverse the wasting process. Despite a falling caloric intake, patients with cachexia frequently show an elevated resting energy expenditure as a result of increases in Cori cycle (i.e., catalytic conversion of lactic acid to glucose) activity, glucose and triglyceride-fatty acid cycling, and gluconeogenesis. A number of cytokines, including tumor necrosis factor- α , interleukins 1 and 6, interferon γ , and leukemia-inhibitory factor, have been proposed as mediators of the cachectic process. However, the results of a number of clinical and laboratory studies suggest that the action of the cytokines alone is unable to explain the complex mechanism of wasting in cancer cachexia. In addition, cachexia has been observed in some xenograft models even without a cytokine involvement, suggesting that other factors may be involved. These probably include catabolic factors, which act directly on skeletal muscle and adipose tissue and the presence of which has been associated with the clinical development of cachexia. A polyunsaturated fatty acid, eicosapentaenoic acid, attenuates the action of such catabolic factors and has been shown to stabilize the process of wasting and resting energy expenditure in patients with pancreatic cancer. Such a pharmacologic approach may provide new insights into the treatment of cachexia. [J Natl Cancer Inst 1997;89:1763-73]

The word "cachexia" is derived from the Greek "kakos" meaning "bad" and "hexis" meaning "condition." It occurs in a number of disease states, including cancer, acquired immunodeficiency syndrome (AIDS), major trauma, surgery, malabsorption, and severe sepsis. Cachexia is characterized by weight loss involving depletion of host adipose tissue and skeletal muscle mass. Weight loss in cancer patients differs from that found in simple starvation. During the first few days of starvation, glucose utilization by the brain and erythrocytes necessitates depletion of liver and muscle glycogen and an increased glucose production by the liver, using gluconeogenic amino acids derived from catabolism of muscle. This early phase is replaced in long-term starvation by the use of fat as a fuel, in which free

fatty acids released from adipose tissue are converted into ketone bodies, which are utilized for energy by peripheral tissues and eventually to a great extent by the brain. This leads to conservation of muscle mass. In anorexia nervosa, more than three quarters of the weight loss arises from fat and only a small amount from muscle. In contrast, in cancer cachexia, there is equal loss of both fat and muscle, so that for a given degree of weight loss there is more loss of muscle in a patient with cachexia than in a patient with anorexia nervosa (1). Thus, although anorexia is common in cancer patients, with reports of occurrence in 15%–40% of subjects at presentation (2), the body composition changes suggest that anorexia alone is not responsible for cachexia. Also, in malnourished cancer patients, the measured food intake fails to correspond with the degree of malnutrition (3), and loss of both muscle and adipose tissue has been reported to precede the fall in food intake (4). In contrast to simple starvation, it is not possible to reverse the body composition changes seen in patients with cancer cachexia by the provision of extra calories. Attempts to increase energy intake in cancer patients through dietary counseling failed to reverse the cachexia (5). Trials of total parenteral nutrition in cachectic cancer patients also failed to show benefit in terms of increased median survival time or long-term weight gain (6). Although a short-term weight gain was observed, this weight was subsequently lost, suggesting the retention of water (7). Analysis of body composition indicated that patients receiving total parenteral nutrition temporarily maintained body fat stores, but there was no evidence for preservation of lean body mass. Thus, the cause of wasting in cancer cachexia is more complex than that in simple starvation.

Anorexia and Cachexia

Although anorexia alone is unlikely to be responsible for the wasting seen in cancer patients, it may be a contributing factor. In addition, its presence is an extremely distressing syndrome because appetite and the ability to eat have been reported to be the most important factors in the physical and psychological aspects of a patient's quality of life (8). Anorexic cancer patients often report early satiety, which together with a reduced appetite has been postulated to be caused by the production of factors by the tumor that exert their effects by acting on the hypothalamic

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sensory cells. Possible candidates for such a factor are the satietyins (9). Satietyins have been purified from human plasma and found to consist of two proteins that copurify until they are purified from one another by affinity chromatography. The larger protein has been characterized as an extensively glycosylated α 1-acid-glycoprotein of a molecular mass of 64 kD, which is probably a vehicle for satietin D, a 41-kD glycoprotein. When injected into rats, satietin D has been shown to produce a long-lasting anorectic effect, although its role in the development of anorexia is not known. There may also be some dysfunction of the hypothalamic neuropeptide Y feeding system in the tumor-bearing state, since rats bearing a methylcholanthrene-induced sarcoma were found to be refractory to the intrahypothalamic injection of neuropeptide Y; normal rats, by contrast, increased their food intake by more than 50% in response to such an injection (10).

Increased serotonergic activity within the central nervous system has been proposed as a possible cause of anorexia. Such activity is secondary to the enhanced availability of tryptophan to the brain. Thus, a close relationship between elevated plasma-free tryptophan and anorexia was observed in patients with cancer and reduced food intake (11). The uptake into the brain of tryptophan is competitive with that of branched chain amino acids. An attempt to reduce tryptophan uptake by increasing plasma levels of competitor branched chain amino acids produced a decrease in the incidence of anorexia (12), but it was not reported whether such patients also regained body weight. However, although the serotonin antagonist cyproheptadine has been reported to have a weight-enhancing effect in normal subjects, a randomized, placebo-controlled, double-blinded trial found it to have no effect on progressive weight loss in cachectic cancer patients (13).

Metabolism in Cancer Cachexia

Metabolic Rate in Cancer Cachexia

In chronic starvation, the basal metabolic rate is reduced as the body adapts to conserve tissues and energy in a low-protein-low-calorie environment (14). However, when compared with control groups, cancer patients have been reported to have reduced (15), normal (16), or increased (17) energy expenditures. Because cancer patients typically have a reduced caloric intake, even a normal energy expenditure could be classified as being in excess. In one study of cancer patients with solid tumors, the basal metabolic rates of the patients were found to be elevated even before the onset of weight loss (18), thus suggesting that an elevated basal metabolic rate may be a contributing factor rather than a consequence of the condition. The elevated basal metabolic rate has been shown to be associated with an increase in heart rate, and it has been suggested that it may be due to an elevated adrenergic state (18). Certainly, many cancer patients show elevated plasma concentrations and increased urinary excretion of adrenergic substances (19), in contrast to malnourished patients without cancer who generally show a decrease in catecholamine turnover.

The tumor type appears to play an important role in determining whether or not an elevation of resting energy expenditure is observed. Thus, patients with lung cancer show an increase in resting energy expenditure compared with that in healthy control

subjects, whereas patients with gastric or colorectal cancer show no elevation in resting energy expenditure (20). Patients with pancreatic cancer have also been reported to have an increased resting energy expenditure compared with that in control subjects, and this effect was found to be more pronounced in those patients with an acute-phase response (21). This may explain why, in a mixed group of cancer patients with gastrointestinal, gynecologic, or genitourinary cancer, 33% were found to be hypometabolic, 41% had average metabolic rates, and 26% were hypermetabolic (15). It has been calculated that an elevation of 12% in the metabolic rate could account for the loss of 1–2 kg of body weight per month (17). Brown adipose tissue plays a thermoregulatory role and has been implicated as an important effector of both body temperature and energy balance in many mammals as well as in humans. Weight loss in a rat model of cancer cachexia has been attributed to a high metabolic rate produced by the activity of brown adipose tissue (22), although results from sarcoma-bearing mice with weight loss suggested that brown adipose tissue could not account quantitatively for the host wasting (23). Results in humans are sparse, although an examination of autopsy samples of periaxillary tissue by light microscopy revealed brown adipose tissue in 80% of cachectic cancer patients compared with 13% of age-matched control subjects (24). The reasons for these changes in basal metabolic rate and brown adipose tissue in patients with cancer cachexia are unknown, although both tumor and host factors, together with the various aberrations in metabolic processes observed in this group of cancer patients, probably contribute to this condition.

Carbohydrate Metabolism

Most solid tumors, either because of isoenzyme alterations or because of their poor vascularization and hence hypoxic nature, rely almost exclusively on the anaerobic metabolism of glucose as their main energy source, with most being converted to lactate (25). Glucose uptake and lactate release by human colon carcinomas have been found to exceed the peripheral tissue exchange rate by 30-fold and 43-fold, respectively (26). Selective transcriptional regulation of hexokinase isoforms by a tumor may enable it to have a growth advantage over normal cells. Thus, the type II isoform has been shown to be the dominant form expressed in AS-30D hepatoma cells in contrast to the type IV isoform in normal hepatocytes (27). The promoter activity of the type II hexokinase was found to be resistant to normal hormonal control, thus enabling tumor cells to maintain glycolysis at an optimal rate regardless of the metabolic state of neighboring healthy cells.

Since glycolysis is an inefficient method of energy production from glucose compared with oxidative phosphorylation, high levels of glucose will be consumed by the tumor. In mice bearing transplantable colon tumors, glucose utilization by the tumor was second only to that by the brain (28). This extra demand for glucose by the tumor was accompanied by a marked decrease in glucose utilization by host tissues, in particular the brain, which resembles the situation found in starvation. Despite this weight loss, cancer patients show a 40% increase in hepatic glucose production compared with control subjects, in contrast to the reduced level seen in patients with anorexia nervosa (29). The increase in glucose production in some cancer patients can be accounted for by an increase in Cori cycle activity (30).

Patients with progressive cancer have been shown to have an increased glucose synthesis not only from lactate (31), but also from alanine (32) and glycerol (33). The Cori cycle normally accounts for 20% of glucose turnover, but it was shown to be increased to 50% in cachectic cancer patients, accounting for the disposal of 60% of the lactate produced (34). Both glucose production rates and recycling rates were found to be higher in malnourished cancer patients than in patients without cancer and with comparable weight loss (35). The increased glucose recycling equivalent to 40% of the daily glucose intake of the cancer patient has been estimated to lead to a potential loss of 0.9 kg of body fat per month.

Hypoglycemia has been reported in a number of animal models of cancer, and in humans it is associated with carcinoma of the stomach, cecum, or bile ducts, pseudomyxoma, and paraganglioma, sometimes occurring before the presence of a tumor is suspected (36). It was originally thought that hypoglycemia was due to the high glucose consumption by the tumor, but a recent study (37) suggests that it arises through the ability of some tumors, other than insulinoma, to produce insulin or insulin-like substances. Since insulin levels have been shown to be low in cases of tumor-associated hypoglycemia, the production by the tumor of an insulin-like growth factor has been suggested as the cause of enhanced peripheral glucose uptake. The most likely candidate for the pathogenesis of extrapancreatic tumor hypoglycemia is the production of a high-molecular-mass (15–25 kd) insulin-like growth factor II (IGF II) by the tumor (37).

Thus, patients with cancer have an increased glucose production and turnover, which may be enhanced by the production of IGF II. Such changes contribute to an increased energy expenditure by the host.

Lipid Metabolism

Fat constitutes 90% of the adult fuel reserves, and loss of whole-body fat is a feature of cancer cachexia. Cancer patients with weight loss have an increased turnover of both glycerol and fatty acids when compared with patients without weight loss (38). Fasting plasma glycerol concentrations have been shown to be higher in weight-losing cancer patients than in weight-stable cancer patients, thus providing evidence for an increase in lipolysis (39). Increased utilization of fatty acids as a preferred energy source has been observed even in the presence of high plasma glucose concentrations, suggesting that, in the presence of certain tumors, host tissues may increase their utilization of fatty acids as an energy source (40).

Several clinical studies [reviewed in (41)] have observed an increased mobilization of fatty acids before weight loss occurs, suggesting the production of lipid-mobilizing factors either by the tumor or by host tissues. Although normal individuals suppress lipid mobilization with administration of glucose, there is an impaired suppression in patients with malignant diseases as well as continued oxidation of fatty acids (42). Increased fatty acid oxidation in the absence of increased dietary fat intake would result in a depletion of fat stores, while increased triglyceride fatty acid cycling and gluconeogenesis from glycerol could result in an increase in metabolic rate. All of these processes, therefore, have the potential to contribute to a net loss of body weight.

Mobilization of fatty acids from host adipose tissue may be

an important factor contributing to tumor growth. Patients with ovarian or endometrial tumors were found to have lower concentrations of linoleic acid in subcutaneous adipose tissue than cancer-free subjects, suggesting mobilization to supply lipids to the tumor (43). Linoleic acid has been found to act as a stimulator of tumor growth both *in vitro* (44) and *in vivo* (45). The effect is probably due to formation of prostaglandins or products of the lipoxygenase pathways. Rat Walker 256 carcinosarcoma cells transfected with 12-lipoxygenase-specific antisense oligonucleotide or antisense oligonucleotides directed to conserved regions of lipoxygenases underwent time- and dose-dependent apoptosis (46). Also, treatment with lipoxygenase but not with cyclooxygenase inhibitors induced apoptotic cell death, which could be partially inhibited by exogenous 12(S)- or 15(S)-hydroxyeicosatetraenoic acids (46). This observation suggests that essential fatty acids from adipose tissue may be required for tumor expansion by preventing cell death by apoptosis.

Protein Metabolism

Lean body mass and visceral protein depletion are characteristics of patients with cancer cachexia, and the degree of depletion may be associated with reduced survival (47). The major site of this protein loss has been observed to be the skeletal musculature (48). An increased rate of whole-body protein turnover has been reported in cachectic cancer patients. A reduced rate of protein synthesis and an increased rate of degradation were observed in muscle biopsy specimens from 43 newly diagnosed cancer patients with weight loss (49). An increase in cathepsin D activity was observed in biopsy specimens from the rectus abdominal muscle, an increase that was associated with the rate of protein degradation. In another study (50), a decrease in muscle protein synthesis was also observed in weight-losing cancer patients, with no change in total body synthesis or degradation. Muscle protein synthesis accounted for only approximately 8% of the total body synthesis in these patients compared with 53% for healthy control subjects. The observed maintenance of the total protein synthetic rate in these patients may therefore be due to a twofold increase in nonskeletal muscle protein synthesis. This twofold increase in protein synthesis may be due to increased hepatic synthesis of secretory proteins such as acute-phase reactants. An elevated whole-body protein turnover may also be apparent in patients with small tumor burdens (51).

There are also changes in the plasma amino acid profile in patients with cancer cachexia, and most studies have reported that such patients exhibit decreases in the concentrations of gluconeogenic amino acids. This result contrasts with the situation in subjects with severe malnutrition, in whom the concentrations of branched chain amino acids in plasma are normal or increased. Compared with normal colon tissue, human colon tumors were observed to have a specific requirement for serine and for the branched chain amino acids valine, leucine, and isoleucine (52). However, the two tissues had similar retention of total amino acids. Correlations between histidine-induced, enhanced formiminoglutamic acid excretion, elevated basal metabolic rate, and reduced serum albumin levels were observed in a study of eight weight-losing patients with small-cell carcinoma of the lung (53). Formiminoglutamic acid excretion was reduced in patients who showed a positive response to chemotherapy,

whereas there was no change in a patient with progressive disease.

The pathway responsible for breakdown of myofibrillar proteins is the adenosine triphosphate (ATP)-ubiquitin-dependent proteolytic system, which has been shown to be elevated in starvation (54), sepsis (55), and metabolic acidosis (56) and after transplantation of certain tumors such as the Yoshida ascites hepatoma in rats (57). In this process, proteins for degradation are first conjugated with ubiquitin, which serves as a signal for degradation by a large proteolytic complex, the 26S proteasome, which requires ATP to function. In skeletal muscle of rats bearing the Yoshida ascites hepatoma, a 500% increase in expression of polyubiquitin genes was observed in relation to both pair-fed (i.e., non-tumor-bearing rats fed the same amount of food as tumor-bearing rats) and *ad libitum*-fed animals (57). Treatment with clenbuterol suppressed the elevation of protein breakdown rates toward control values, and this suppression was associated with a decreased expression of polyubiquitin genes (58). Thus, understanding more about this proteolytic system and factors involved in its regulation may provide important clues for the treatment of muscle wasting in cachexia.

Factors Implicated in Production of Cancer Cachexia

Numerous cytokines, including tumor necrosis factor- α (TNF- α), interleukin 1 (IL-1), interleukin 6 (IL-6), interferon gamma (IFN γ), and leukemia-inhibitory factor (LIF), have been postulated to play a role in the etiology of cancer cachexia. Such cytokines may be produced by tumor or host tissue and are characterized by the induction of anorexia and a decrease in the clearing enzyme lipoprotein lipase. The ability to inhibit lipoprotein lipase varies among the cytokines. Thus, while LIF is twofold to 10-fold less potent than TNF- α , it is 100 times more potent than IL-6 (59). However, it is unlikely that a decrease in lipoprotein lipase alone could account for the fat cell depletion and wasting seen in cachexia, since in type 1 hyperlipidemia caused by an inherited deficiency in lipoprotein lipase, patients have normal fat stores and are not cachectic. This fact, together with the inability of the cytokines to explain all of the metabolic changes associated with cancer cachexia, has inspired investigators to search for tumor-produced catabolic factors that act directly on adipose tissue and skeletal muscle initiating the process of cachexia.

Tumor Necrosis Factor- α (TNF- α)

TNF- α was suggested as a possible cachectic factor as a result of studies on the mechanism of the weight loss observed in rabbits chronically infected with *Trypanosoma brucei brucei*. Administration of TNF- α to laboratory animals induces a state of cachexia, with anorexia and depletion of adipose tissue and lean body mass. However, tachyphylaxis rapidly develops following administration of additional TNF- α (60). Tachyphylaxis was not found to develop in immunodeficient nude mice that were given an injection of Chinese hamster ovary (CHO) cells transfected with the human TNF- α gene (61). These cells induced high serum levels of TNF- α (1.0–22.8 ng/mL) in the mice and a syndrome resembling cancer cachexia with progressive wasting, anorexia, and early death. It is interesting that, when

such cells were transplanted intracerebrally, hypophagia and weight loss were observed, and the body composition was comparable to that seen in starvation, i.e., a decrease in whole-body lipid but conservation of protein (62). When the tumor cells were transplanted intramuscularly, profound anorexia did not develop; after a long period of tumor burden (50 days), however, cachexia developed and both protein and lipid were depleted.

In addition to inhibition of lipoprotein lipase (59), incubation of cultured human fat cells with TNF- α has been shown to induce a marked dose-dependent stimulation of lipolysis by up to 400% of control values; this stimulation became apparent after a 6-hour exposure at the earliest (63). TNF- α has been shown in several studies (64–68) to activate muscle protein degradation, although not all of the reports agree that TNF- α does activate protein degradation directly. In one of the studies (65), TNF- α administration to healthy (i.e., cancer-free) rats brought about an enhanced rate of degradation of skeletal muscle protein, even though body weight loss was not apparent in the animals. In another of the studies (68), administration of anti-murine TNF- α immunoglobulin to rats bearing the Yoshida AH-130 ascites hepatoma led to decreases in the rates of protein degradation in the skeletal muscle, heart, and liver tissues, but it had no effect on weight loss in the animals. However, a direct action of TNF- α has not been demonstrated by most authors, when either tyrosine or 3-methylhistidine was used as a measure of the proteolytic rate (69). Despite this result, a recent report (67) has shown an increase in ubiquitin gene expression, with no change in the expression of the C8 proteasome subunit after incubation with TNF- α for 180 minutes *in vitro*. These results show that TNF- α has the potential to act as a modulator of the cachectic process.

Some animal tumors are thought to produce the cachectic syndrome through the mediation of TNF- α . The Yoshida AH-130 ascites hepatoma induces weight loss in the host, and elevated endogenous circulating TNF- α and prostaglandin E₂ levels are observed (68). Administration of anti-TNF- α antibody before transplantation of the tumor abolished detectable TNF- α levels, while fractional rates of protein degradation in gastrocnemius muscle, heart, and liver were reduced, although treatment failed to prevent a reduction in body weight. A similar result was obtained in mice with methylcholanthrene-induced sarcomas, where anti-TNF- α monoclonal antibody delayed but did not prevent the development of anorexia and had no effect on overall body weight (70). In another model, the Lewis lung adenocarcinoma anti-TNF- α antibody partially reversed the loss of body fat, without affecting food intake or causing a change in body weight (70). This finding suggests that TNF- α may not be responsible for the changes seen in animals or humans with cancer cachexia.

Similar problems have been encountered in clinical studies on the role of TNF- α in cancer cachexia. While a short intravenous infusion of recombinant human TNF- α increased plasma triglyceride levels, glycerol turnover by more than 80% and free fatty acid turnover by more than 60% (71,72), in the case of long-term administration, the changes resolved despite the continuous administration of TNF- α (71). Similar effects were seen on protein metabolism, where whole-body protein turnover was increased as measured by ¹⁵N enrichment of urinary urea and ammonia (71). Thus, TNF- α has the potential to induce catabolism of

adipose tissue and skeletal muscle in humans, although anorexia has not been reported to be a major dose-limiting toxicity. Several studies have failed to detect elevated circulating levels of TNF- α in cachectic cancer patients (73,74) or have failed to associate the elevation with the development of cachexia (75). Elevation of serum TNF- α levels seems to be associated more with the clinical status of the patient. Thus, in a study of 91 patients with B-cell chronic lymphocytic leukemia, serum levels of TNF- α were elevated in all stages of the disease with a progressive increase in relation to the stage (76). TNF- α levels were found to be substantially higher in patients with endometrial carcinoma than in healthy postmenopausal women or in women with endometrial hyperplasia, and serum TNF- α levels were associated with advancing stage of disease (77). Increased serum levels of TNF- α receptors were found in a range of solid tumor types, and the incidence and extent of the increase also were associated with the staging of the disease (78).

The inability to associate serum levels of TNF- α with the development of cachexia may be due to the very rapid blood transit of cytokines, so that they can be transported from the sites of production to the target tissues without causing an elevated serum concentration. However, elevated concentrations of TNF- α have been reported in patients with both malaria (79) and visceral leishmaniasis (80), which has been associated with death and cachexia. In addition, although animal experiments have shown TNF- α to be a potent inhibitor of lipoprotein lipase (59), there appears to be no difference between cancer patients and control subjects in either the total lipoprotein lipase activity or the relative levels of the messenger RNA (mRNA) for lipoprotein lipase and fatty acid synthesis (74), thus raising a question of a local effect for this cytokine. In rats infected with *Escherichia coli*, pentoxifylline (a potent inhibitor of TNF- α secretion) decreased the anorexia, loss of body weight, and muscle protein observed and partially prevented the decrease in muscle protein synthesis induced by infection (81). However, in a clinical study (82), pentoxifylline failed to reduce anorexia or cachexia in 35 patients with cancer.

Interleukin 6 (IL-6)

A potential role for IL-6 in the development of cancer cachexia has mainly been provided from animal studies involving the use of the murine colon-26 adenocarcinoma model (83-85), in which increasing levels of IL-6 appear to lead to the development of cachexia (83). In addition, the administration of anti-mouse IL-6 monoclonal antibody, but not of anti-mouse TNF- α monoclonal antibody, attenuated the development of weight loss and other parameters of cachexia in the mice (83). In another study (84) in which clonal variants of the colon-26 tumor model were used, the serum concentrations of IL-6 in mice bearing a tumor clone that does not induce weight loss were lower than in mice bearing a tumor clone that does induce weight loss; however, infusion of IL-6 into mice in the former group did not lead to body weight loss. These results indicate that IL-6 was not solely responsible for the induction of cachexia. Suramin, a polysulfonated naphthylurea, has been shown to inhibit the binding of IL-6 to cell surface receptor subunits, and it has been shown to partially block cachexia in the colon-26 model, without a decrease in tumor burden (85). Since anti-IL-6 antibody treatment did not enhance the effect, this result suggests that suramin

inhibits cachexia, at least in part, by interfering with the binding of IL-6 to its receptor. IL-6 has also been identified as a mediator of cachexia by the growth of a uterine cervical carcinoma called Yomoto in nude mice (86). Administration of a neutralizing antibody against human IL-6 to mice, after the development of cachexia, was shown to reduce the loss of body weight and the wasting of adipose tissue (86).

Agents that regulate cytokines, such as interleukin 12 (IL-12), reduced the serum levels of IL-6 in mice bearing the murine colon-26 carcinoma and alleviated the loss of body weight, adipose tissue wasting, and hypoglycemia associated with cachexia (87). IL-12 also substantially increased IFN γ levels in the tumor, and IFN γ administered intraperitoneally also prevented the cachexia, although it did not reduce IL-6 levels. It is interesting that IFN γ has been reported to be a possible mediator of cachexia in other animal model systems (88). Interleukin 10 (IL-10) was originally identified as a cytokine synthesis inhibitory factor. A reduction in serum IL-6 levels was observed in mice bearing the colon-26 tumor transfected with the IL-10 gene, although these levels did not reach baseline values and such mice did not develop cachexia (89). Since cachexia was completely prevented with an incomplete reduction of serum IL-6 levels, the authors suggested that an additional unknown factor was responsible for the development of cachexia in this model. This suggestion was also made in another study (90) on mice bearing the colon-26 tumor. An antibody to the IL-6 receptor reduced the loss of weight of the gastrocnemius muscle and suppressed the enzymatic activity of cathepsins B and L and mRNA levels of cathepsin L and poly-ubiquitin, but it had no effect on the overall loss of body weight or wasting of adipose tissue, suggesting that the latter two may not be influenced by IL-6.

Certainly, IL-6 has the potential to act as a cachectic factor. Atrophy of muscles is observed in IL-6 transgenic mice; this atrophy is completely blocked by anti-mouse IL-6 receptor antibody (91). The muscle atrophy is associated with increased mRNA levels for cathepsins (B and L) and mRNA levels of ubiquitins (poly- and mono-). Administration of IL-6 to rats has also been shown to acutely activate both total and myofibrillar protein degradation in skeletal muscle (92). In an *in vitro* study (93) using murine C₂C₁₂ myotubes grown in cell culture, exposure of the cells to 100 U/mL recombinant human IL-6 was found to shorten the half-life of long-lived proteins and to increase the activity of the 26S proteasome and lysosomal (cathepsins B and L) proteolytic pathways. This result suggests that IL-6 is capable of directly up-regulating pathways of protein degradation.

Unlike the results obtained with TNF- α , statistically significant increases in IL-6 and C-reactive protein, as a measure of the acute-phase response, have been found in weight-losing patients with non-small-cell lung cancer, when compared with patients with the same tumor, but without weight loss (94). An elevated level of serum IL-6 has also been reported in patients with colon cancer and an acute-phase response (95); however, since all patients had lost weight, it is difficult to associate this elevation with the induction of cachexia.

The results of these animal and human studies strongly implicate IL-6 in the cachectic process. However, IL-6 probably

does not act alone but may either induce or act in synergy with other cachectic factors.

Interleukin 1 (IL-1)

IL-1 has been shown to have many effects similar to those of TNF- α ; these similar effects include suppression of lipoprotein lipase and enhancement of intracellular lipolysis. Administration of recombinant IL-1 was observed to induce anorexia, weight loss, hypoalbuminemia, and elevated amyloid P levels in the mouse (96). Indeed, this cytokine was observed to have a greater anorexigenic effect than TNF- α when administered in isomolar quantities. However, in the MCG 101 sarcoma tumor model of cachexia in mice, IL-1 mRNA was present at low levels as determined by northern blot analysis in the spleen, liver, intestine, and brain and at elevated levels in the spleen (97). IL-1 and TNF- α protein were both detected in tumor tissue, but at levels similar to those in normal tissue. Neutralizing antibodies against the IL-1 receptor were observed to cause statistically significant, but minor, inhibitory effects on cachexia and anorexia in this model (98). Administration of the IL-1 receptor antagonist to rats bearing the Yoshida ascites hepatoma was also found to be completely ineffective in preventing tissue depletion and protein hypercatabolism (99). Transfection of a cachectic tumor cell line (colon-26) with the gene for the IL-1 receptor antagonist also failed to abolish the capacity of the tumor to produce cachexia (100). These results cast doubt on a role of this cytokine in the induction of tissue wasting in cancer cachexia.

Interferon Gamma (IFN γ)

Interest in the role of IFN γ in the pathogenesis of cancer cachexia developed as a result of observations confirming that it had properties similar to those of TNF- α with respect to fat metabolism *in vitro* (101). Weight loss in mice bearing the Lewis lung tumor is associated with IFN γ production, and administration of an anti-IFN γ antibody reduced the depletion of body fat but had no effect on total body protein (102). In rats that had received transplants of the MCG 101 sarcoma, anti-IFN γ antibody reduced weight loss and anorexia and increased survival, but the treatment was partial and short-lived, suggesting that IFN γ may not be the sole mediator (103). Inoculation of CHO cells transfected with the IFN γ gene into mice resulted in a dose-related development of anorexia and marked weight loss due to fat and muscle depletion, not wholly attributable to the reduction in food intake (104). Such a result should not be interpreted to mean that IFN γ by itself can induce cachexia, since both IFN γ release and the presence of the tumor cells were found to be required. While serum TNF- α levels of patients with multiple myeloma did not differ from those found in healthy control subjects, IFN γ was found to be raised in 53% of the patients (105). However, no association was observed between the level of IFN γ and clinical parameters of the disease. These results suggest that IFN γ alone may not be responsible for the induction of cachexia.

Leukemia-Inhibitory Factor (LIF)

LIF has also been suggested to play a role in the cancer cachexia syndrome through its ability to decrease lipoprotein lipase activity. Nude mice implanted with the human cell line

SEKI, which expresses large amounts of LIF, develop a severe cachexia (106). LIF mRNA has also been shown to be present in two types of melanoma xenograft that induce weight loss in transplanted animals, whereas none was detected in non-cachexia-inducing xenografts (106). Although inhibition of lipoprotein lipase has been suggested to account for the cachectic effect of LIF, it is unlikely that a decrease in lipoprotein lipase alone could account for the fat cell depletion, and no mechanism has been proposed to explain skeletal muscle catabolism. It seems unlikely that any of the cytokines alone are able to explain the complex mechanism of wasting seen in cancer cachexia, and other factors must be involved. This view is substantiated in a study of the factors responsible for the cachectic syndrome in nude mice bearing human tumor xenografts (107). In four of the eight models, a cytokine such as LIF or IL-6 produced by the cancer cells may be responsible; however, in the remaining four cancer cell lines, the inducing factor was unknown. In these cases, the inducing factors may be catabolic factors, which act directly on host tissues.

Lipid-Mobilizing Factors (LMFs)

LMFs act directly on adipose tissue with the release of free fatty acid and glycerol in a manner similar to that of the lipolytic hormones. Like the induction by hormones, the induction of lipolysis is associated with an elevation of intracellular cyclic adenosine monophosphate (108), possibly as a result of stimulation of adenylate cyclase. Evidence for the production by tumors of an LMF was provided by Costa and Holland (109), who showed that nonviable preparations of the Krebs-2 carcinoma, when injected into mice, were able to induce the early, rapid stage of fat depletion, which represented true cachexia in this model. Ascites serum from rats transplanted with the Walker 256 carcinoma increased stimulation of lipolysis in an *in vitro* assay (110), whereas serum from mice bearing a thymic lymphoma when injected into non-tumor-bearing controls produced massive fat loss (111), providing further evidence for an LMF. This latter factor was also detected in extracts of the tumor, in tissue culture medium, and in the sera of patients with adenocarcinomas of the cervix and stomach, thus suggesting that the LMF was tumor derived and circulatory. Other studies have shown that the level of LMF in the sera of cancer patients was proportional to the extent of weight loss (112) and was reduced in patients responding to chemotherapy (113).

Most studies provide evidence that the LMF is an acidic protein, although there appears to be variations in the molecular weight. A heat-stable protein of molecular mass around 5 kd was isolated from a thymic lymphoma (114), and another heat-stable protein of molecular mass 6 kd was isolated from the conditioned medium of the A375 human melanoma cell line (115). A heat-labile material of molecular mass between 65 kd and 75 kd was isolated from the ascites fluid of patients with hepatoma and mice with sarcoma 180 (116). Tryptic digestion of the active material produced a low-molecular-weight material that was still active.

These studies provide strong evidence for the production of LMF by tumors. The production of this material appears to be related to the process of cachexia, since LMF is absent or present in reduced amounts in tumors that do not induce cachexia (117) and is absent from normal serum, even under conditions of star-

vation (118). No report to date has provided sequence information on these LMFs; therefore, further studies are required.

Protein-Mobilizing Factors (PMFs)

Using bioassays to detect protein degradation, investigators have found evidence for the existence of PMF(s) in the sera of both animals (119) and humans (120) with cancer cachexia. The bioactivity appears to be associated with the loss of skeletal muscle mass and is absent from the sera of healthy control subjects. This material has now been purified from a cachexia-inducing murine tumor (MAC16) and from the urine of patients with cancer cachexia by use of affinity chromatography with a monoclonal antibody derived from mice bearing the MAC16 tumor (121). The PMF from both murine and human sources appeared to be identical and was characterized as a sulfated glycoprotein of a molecular mass of 24 kd and of unique amino acid sequence (122,123). Although the PMF was readily detected in the urine of cachectic cancer patients, irrespective of the tumor type, it was absent from the urine of cancer patients with little or no weight loss, from the urine of normal subjects, or from the urine of patients with weight loss due to trauma or sepsis (122). When the PMF was injected into non-tumor-bearing mice, rapid weight loss (about 10% in 24 hours) was observed, without a reduction in food and water intake, and body composition analysis showed selective depletion of the lean body mass. Evidence for a direct effect of the PMF was provided by the induction of protein degradation in isolated gastrocnemius muscles (121). The conservation in structure of this material between murine and human sources suggests that production of a PMF may be important in the growth and survival of some tumors.

Treatment of Cancer Cachexia

Weight loss is associated with psychologic distress and a lower quality of life. In addition, patients with weight loss have a shorter survival time and a decrease in response to therapy (124). About half of all patients with cancer show some weight loss (124), but those with pancreatic cancer show it at the highest frequency (125); in the latter study, the investigators found that all patients at the time of diagnosis had lost weight (median, 14.2% of pre-illness stable weight), and this weight loss was progressive, increasing to a median of 24.5% just before death. Patients with more than 15% weight loss are likely to have substantial loss of total body protein, and at this level of lean tissue depletion, physiologic function (e.g., respiratory muscle function) is markedly impaired (126). Thus, such patients need effective therapy if death from cachexia is not to occur.

As previously discussed, nutritional support in the form of total parenteral nutrition has failed to replete lean body mass. Even worse, a meta-analysis of the published trials on patients receiving total parenteral nutrition while undergoing chemotherapy showed a decreased survival, a poorer tumor response, and a significantly significant increase in infectious complications (127).

An improvement in appetite alone does not fully reverse the cachectic syndrome. Thus, patients with advanced malignant disease receiving medroxyprogesterone acetate (100 mg taken orally three times a day) showed a great improvement in appe-

tite, but this effect did not result in weight gain or an improvement in performance status, energy levels, mood, or relief from pain (128). Results with the appetite stimulant megestrol acetate look more promising in terms of weight gain. A number of clinical studies (129,130,131) have been performed, all of which report an increase in appetite and weight gains of up to 6.8 kg over baseline values in 16% of patients treated. However, body composition analysis, as determined by use of dual-energy x-ray absorptiometry and tritiated body water methodologies measured at the time of maximum weight gain, showed that the majority of patients gained weight from an increase in adipose tissue, while an increase in body fluid was responsible for a small portion of the weight gained (131). An increase in lean body mass was not observed. Such body composition changes are similar to those observed in patients receiving total parenteral nutrition (7).

Pharmacologic approaches to the treatment of cancer cachexia have been more successful. Hydrazine sulfate, an agent that inhibits the enzyme phosphoenolpyruvate carboxykinase, has been demonstrated to favorably influence the abnormal glucose and protein metabolism in cachectic cancer patients (132) and to maintain or even increase body weight (133). Ibuprofen, a cyclooxygenase inhibitor, has been shown to reduce the resting energy expenditure in patients with pancreatic cancer, suggesting that it may have a role in abrogating the catabolic processes that contribute to weight loss (134). Serum C-reactive protein levels were also reduced. The polyunsaturated fatty acid eicosapentaenoic acid, another cyclooxygenase inhibitor, has also been shown to counteract the weight loss in patients with pancreatic cancer with stabilization of protein and fat reserves (135). This result was accompanied by a temporary reduction in acute-phase protein production and stabilization of resting energy expenditure. The effect appears to be specific for eicosapentaenoic acid, since patients receiving a related polyunsaturated fatty acid, gammalinolenic acid, continued to lose weight. A similar structure-activity relationship was observed in mice with cachexia induced by the MAC16 tumor (136). Eicosapentaenoic acid appears to act by attenuating the action of catabolic factors in cachexia. Induction of lipolysis by an LMF was inhibited by eicosapentaenoic acid, and the effect appeared to be due to prevention of the rise in adipocyte cyclic adenosine monophosphate levels (108). Administration of eicosapentaenoic acid also led to statistically significant reductions in protein degradation *in vivo* (136), possibly as a result of the ability to inhibit prostaglandin E₂ production in skeletal muscle by a PMF (119).

Summary

Although cancer cachexia superficially resembles starvation, nutritional intervention alone is unable to reverse the condition. Tremendous progress has been made in the last 10 years in elucidating the role of various factors in host tissue catabolism, and the results of these studies are now being translated into treatment regimens for the benefit of patients with cachexia. Cachexia is an important cause of mortality in cancer patients, accounting directly for between 10% (137) and 22% (138) of all cancer deaths, as well as death from other causes such as infection. Thus, an effective therapy for cachexia not only should improve the quality of life of cancer patients, but also may be

expected to extend the survival time. In addition, since some tumors may depend on the products from host tissue catabolism for survival, such therapy may also have an antitumor effect. Considering that cachexia is common in those cancers for which therapy is currently limited, this could prove to be of great clinical benefit.

Appendix: Methodology

Purpose

Our goal was to review the metabolic processes that contribute to cancer cachexia-related tissue wasting and to critically assess the role of cytokines and catabolic factors as mediators of these processes; studies of this condition in humans were emphasized as much as possible.

Information Source

Our sources of information were Medline (National Library of Medicine, Bethesda, MD), *Current Contents* (Institute for Scientific Information, Philadelphia, PA), and a large reprint file built up over 15 years. No study has been excluded on the basis of the date of publication if the results are still appropriate, although emphasis has been placed on the most recent studies whenever possible. Where several authors report the same result, only one study is included, usually the first published.

Criteria for Evaluating Validity

Data from studies on humans rather than on experimental animals have been used whenever possible, particularly if results from the latter contradict the human evidence. Results from animal experiments that involved use of models not appropriate to the human condition have been excluded; these included studies of tumors that are rapidly growing or where the weight of the tumor was large in relation to the weight of the animal. Since cachexia is an *in vivo* phenomenon, priority was given to studies in which whole animals were used.

Methods for Summarizing Evidence

There was insufficient data in this area to warrant highly structured quantitative techniques, and a simple narrative approach has been used to summarize the evidence.

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Interleukins and tumor necrosis factor as inhibitors of food intake

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Summary Cytokines, such as interleukins and tumor necrosis factor- α (TNF α), are produced in response to immune stimulation and have systemic effects, mediated by the central nervous system (CNS). Interleukins, in particular interleukin [IL]-1 β , and TNF α reduce food intake after peripheral and central administration, suggesting that they contribute to the anorexia during various infectious, neoplastic and autoimmune diseases. Because cytokines are mainly produced in the periphery during most of these diseases, IL-1 β and TNF α may inhibit feeding indirectly through neural and humoral pathways activated by their peripheral actions. Activation of afferent nerve fibers by locally produced cytokines in the periphery is involved in several cytokine effects, but is not crucial for the anorectic effect of systemic immune stimulation. Cytokines increase OB protein (leptin) expression in the adipose tissue, and leptin may contribute to, but is also not essential for, the anorectic effects of cytokines. Finally, circulating IL-1 β and TNF α may act directly on the brain or cytokine synthesis in the brain may contribute to the anorectic effect of systemic immune stimulation. Central mediators of the anorectic effects of cytokines appear to be neurochemicals involved in the normal control of feeding, such as serotonin, corticotropin releasing factor, histamine, α -melanocyte stimulating hormone, and neuropeptide Y. The well-documented cytokine production in the gut in relation to feeding and the expression of TNF α by adipocytes suggest that IL-1 β and TNF α may also play a role in the control of normal feeding and energy balance. All in all, reciprocal, synergistic and antagonistic interactions between various pleiotropic cytokines and between cytokines and neurochemicals form a complex network that mediates the effects of cytokines on feeding and energy balance. © 1999 Harcourt Publishers Ltd

INTRODUCTION

In response to immune stimulation, blood monocytes, tissue macrophages, and a variety of other cells [blood neutrophils, central nervous system (CNS) microglia, astrocytes, endothelial cells, and fibroblasts] produce and release a series of proinflammatory cytokines such as interleukin-[IL]1, IL-6, IL-8, and tumor necrosis factor- α [TNF α]. These cytokines are part of the cytokine network that orchestrates non-specific and specific immune reactions, and causes clear CNS-mediated effects, such as fever, sleep, activation of the hypothalamic-pituitary-adrenal (HPA) axis, and anorexia.¹ All these changes are

beneficial for the organism in the acute phase of an immune reaction.² Long-term anorexia, however, compromises host defense and is ultimately deleterious. Although various cytokines use different transduction mechanisms, many of them have overlapping actions. In addition to their well-known immune functions, cytokines are increasingly recognized as fundamental regulators of metabolism and energy balance. Several cytokines, including IL-1, IL-2, IL-6, IL-8, TNF α and Interferon- γ , inhibit feeding after peripheral or central administration (see ref. 3 for review), suggesting that their enhanced production contributes to anorexia in disease. This review will focus on IL-1 and TNF α because they have the most pronounced effect on feeding. After a brief description of some general features of IL-1 and TNF α and their anorectic effects, we will discuss the potential sites and mechanisms of their action. We will then consider possible functions of these cytokines in normal feeding and body weight regulation.

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CYTOKINE SYSTEMS

The IL-1 system⁴ consists of the biologically active IL-1 β and IL-1 β , the naturally occurring IL-1 receptor antagonist (IL-1Ra), the type I IL-1 receptor (IL-1RI) that is responsible for signaling, and the type II IL-1 receptor (IL-1RII) that inhibits IL-1 by acting as a decoy surface molecule. The system is completed by the IL-1 receptor accessory protein (IL-1R ACP), which increases the binding affinity of IL-1 for IL-1RI, and by soluble forms of IL-1RI, IL-1RII and IL-1R ACP. The biological effects of IL-1 include immune (T- and B-cell activation, lymphokine gene expression, etc.) and pro-inflammatory (fever, sleep, endothelial cell activation, neutrophilia, cyclooxygenase and lipoxygenase gene expression, beta islet cytotoxicity, etc.) properties, as well as protective effects.

The TNF α system includes two types of receptors which are thought to elicit distinct responses and also exist in soluble forms.⁵ TNF α is also a pleiotropic cytokine that has many immune and pro-inflammatory functions and profound metabolic effects. As with the IL-1 system, the biological activity of endogenous TNF α depends on the balance between TNF α and cell surface and soluble TNF α receptors.

Mice genetically deficient in a particular cytokine or cytokine receptor display altered reaction patterns for fever, increases in cytokine mRNA levels, and serum corticosterone,^{6,7} reflecting the key role of cytokines in these functions. In contrast, the anorexia in response to immune stimulation is usually unaltered in genetically cytokine or cytokine receptor-deficient mice. For example, bacterial lipopolysaccharide (LPS), which is often used to model infection, reduces food intake similarly after parenteral administration in mice genetically deficient in either IL-1 β or TNF α or their receptors.⁸⁻¹¹ On the other hand, rats made tolerant to the anorectic effect of intraperitoneally (IP) injected TNF α did not reduce food intake in response to IP LPS,¹² suggesting that endogenous TNF α plays a role in mediating LPS anorexia. Moreover, the phosphodiesterase inhibitor pentoxifylline attenuated the increases in circulating cytokines (in particular TNF α) as well as the anorexia induced by LPS.¹³ Other studies implicate IL-1 β and/or IL-6 in various models of anorexia.¹⁴⁻¹⁷ Thus, interleukins and TNF α play a prominent role in the anorexia during diseases, and this role becomes evident when cytokines or their actions are acutely antagonized. On the other hand, adaptive and compensatory mechanisms throughout development obviously limit the link between the knockout gene product and anorexia in mice lacking a particular cytokine or a cytokine receptor. This is presumably related to the redundancy¹⁸ and synergistic interactions of cytokines, which are also observed with respect to the anorexia after peripheral and central administration.¹⁹⁻²¹

FOOD INTAKE SUPPRESSION BY INTERLEUKINS AND TNF α

General considerations

The magnitude of the food intake suppression by cytokines depends on the type of the cytokine, the route of administration, and the administered dose. The anorectic effect of IL-1 and TNF α is specific because it is blocked by cytokine receptor antagonists or antibodies,^{17,22,23} and is independent of the reduction in water intake,^{24,25} fever, and sleep-promoting effects of these cytokines (see refs 22, 25). Further, although taste aversions can be produced by peripheral administration of high doses of cytokines, they do not play a major role in IL-1 β and TNF α -induced anorexia.²⁶⁻²⁸ In a macronutrient choice situation, cytokines specifically reduce protein intake.²⁹ Finally, some studies suggest that cytokine-induced anorexia depends on the energy state of the body.^{30,31} Thus, IL-1 or experimental inflammation failed to reduce food intake in rats after prior body weight reduction, and all rats achieved the same level of body weight independent of test onset body weight. Based on these data, the authors proposed that cytokines may cause a temporary reduction in body weight set point that is spontaneously maintained by reduced feeding.³⁰

Chronic peripheral administration of IL-1 and TNF α is often accompanied by tolerance to their anorectic effects (e.g. refs 12, 30, 32, 33). Yet, tolerance is not observed with continuous intracerebroventricular (ICV) infusion of IL-1 β ³¹ or with repetitive IP injections of IL-1 β , given when the anorectic effect of the previous injection has subsided.³² In the latter situation sensitization occurs, i.e. the anorectic effect of IL-1 β is enhanced. This is interesting because cytokines may be released in a cyclic fashion during several diseases.

Food intake is reduced after ICV administration of cytokines at doses in the picogram-nanogram range that yield pathophysiologically relevant concentrations in the CSF.^{21,33} Much higher doses have to be administered peripherally to reduce food intake. This indicates that centrally administered cytokines inhibit feeding by a direct action in the brain. Overproduction of cytokines in the brain may contribute to the anorexia during some diseases that specifically target the CNS.^{32,34} A role of central cytokine action has also been suggested for the anorexia in response to acute colitis induced by rectal administration of trinitrobenzenesulfonic acid in the rat, where ICV administration of IL-1Ra attenuated the accompanying inhibition of feeding.¹⁶ In another study,¹⁶ intra-VMH injection of IL-1Ra improved food intake in anorectic tumor-bearing rats, suggesting that centrally acting IL-1 contributes also to cancer anorexia. Yet, exogenous cytokines have different effects on meal patterns after peripheral and central administration:

whereas ICV administered cytokines reduce food intake by primarily reducing meal size,³⁵ IP injected IL-1 β and TNF α have been shown to affect meal size and meal frequency,^{36,37} and IP injected LPS selectively reduces meal frequency.³⁷ Moreover, IL-1 β -converting enzyme-deficient mice resist the anorectic effect of ICV, but not IP administered LPS,³⁸ suggesting that IL-1 β is crucial for the anorectic effect of central, but not peripheral LPS. In line with these findings, ICV administration of IL-1Ra inhibited the effect of ICV injected LPS, but failed to attenuate the food intake suppressive effects of IP LPS injection.³⁹ Finally, repetitive ICV TNF α pretreatment failed to prevent ICV LPS anorexia⁴⁰ indicating that, in contrast to peripheral LPS's anorectic effect,¹² the central anorectic effect of LPS does not depend on TNF α -mediated pathways.

In sum, central and peripheral immune stimulation appear to reduce food intake through activation of different mechanisms, suggesting that central cytokine administration does not necessarily reflect the pathophysiological

mechanisms relevant for systemic diseases. This does not mean that activation of central cytokine systems is not involved in the anorexia induced by peripheral immune stimulation; rather the mode and pattern of activation may be different, or concomitant changes in peripheral input may be crucial to shape the ultimate feeding response.

Peripheral cytokines in anorexia

During most infectious diseases and in response to other challenges of the immune system, interleukins and TNF α are mainly produced in the periphery. To inhibit feeding, these cytokines or mediator(s) or signal(s) derived from their peripheral action(s) must somehow influence the brain circuitry that controls food intake. C-fos studies have shown that peripheral administration of cytokines activates several CNS regions that are involved in the control of eating⁴¹ but the mechanisms of this activation and how this leads to anorexia are unclear. Humoral and neural pathways are probably involved (Fig. 1).

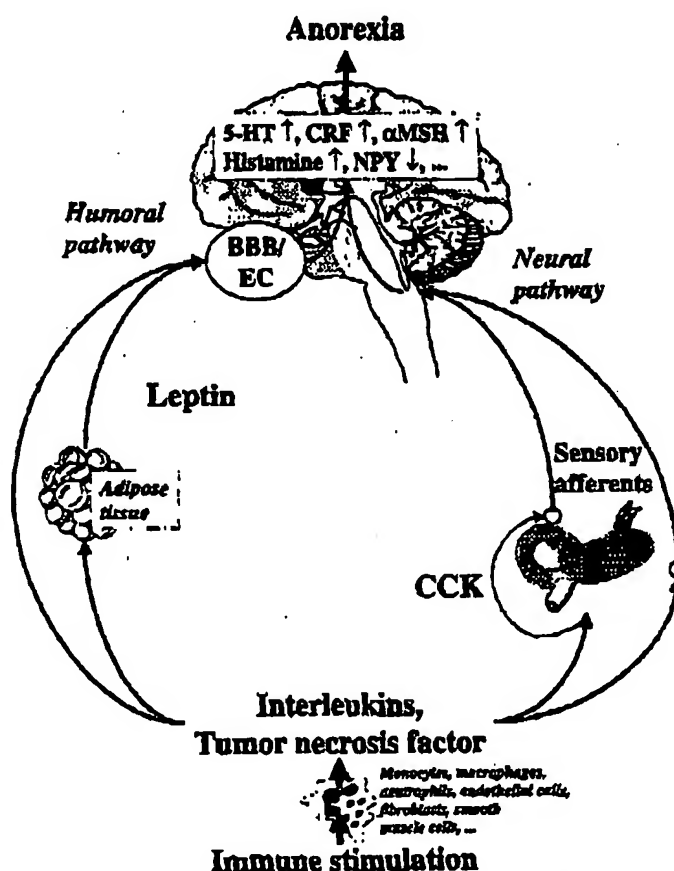


Fig. 1 Possible pathways for the inhibition of feeding by cytokines in response to peripheral immune stimulation. BBB: blood brain barrier; CCK: cholecystokinin; EC: endothelial cells. Increases (↑) in turnover or activity of serotonin (5-HT), α -melanocyte stimulating hormone (α MSH), histamine and corticotropin releasing factor (CRF), and a decrease (↓) in neuropeptide Y (NPY) appear to contribute to the central neurochemical mediation of the inhibition of feeding by cytokines. See text for further details.

Mediation by visceral afferent nerves

Localized peripheral cytokine production, which does not increase circulating cytokine levels, plays a role in several immune functions.⁴² Interleukins and TNF α are released locally (by tissue macrophages, endothelial cells, etc.), at the site of immune stimulation,⁴³ and may activate nearby sensory afferent fibers (Fig. 1). Subdiaphragmatic vagal afferent fibers in rats are responsive to peripheral IL-1 β . Intravenous (IV) injection of IL-1 β increased multiunit activity in the distal cut segment of gastric branch vagal afferent fibers,⁴⁴ and injection of IL-1 β into the hepatic portal vein dose-dependently increased multiunit activity in hepatic branch vagal afferent fibers.⁴⁵

Subdiaphragmatic vagotomy has been reported to attenuate or block a variety of behavioral and neural effects induced by peripheral administration of IL-1 β or LPS, including CNS c-fos expression,⁴⁶ c-fos induction in the nodose ganglion,⁴⁷ induction of IL-1 mRNA in hypothalamus, hippocampus and brainstem,^{48,49} fever,^{50,51} and increases in plasma ACTH⁵² and corticosterone.⁵³ Furthermore, subdiaphragmatic vagotomy attenuated the conditioned taste aversion,⁵⁷ depression of social exploration,⁵⁴ hyperthermia,⁵⁵ and hyperalgesia⁵⁶ induced by peripheral administration of IL-1 β and LPS.

A role for the afferent vagus nerve in the inhibition of food intake by IL-1 β has been suggested because subdiaphragmatic vagotomy attenuated IP IL-1 β -induced suppression of instrumental responses to obtain food in mice.⁵⁷ Subdiaphragmatic vagotomy, however, did not block the effects of subcutaneously or IV administered IL-1 β ,⁵⁴ and in more recent studies, systemic capsaicin pretreatment failed to block the effects of IP administered IL-1 β and LPS on food-motivated behavior.⁵⁸ In our hands, IP injected IL-1 β and LPS still reduced food intake after subdiaphragmatic vagal deafferentation as potently as in sham-operated rats.⁵⁹ Therefore, subdiaphragmatic vagal afferent fibers, despite their putative role in mediation of other immune reactions (see above), are not necessary for the anorectic effects of peripheral IL-1 β and LPS.

Non-vagal, splanchnic afferent pathways have also been implicated in several immune responses.⁶⁰ Yet, surgical transection of the celiac/superior mesenteric ganglion complex failed to attenuate LPS-induced hyperalgesia.⁵⁵ We observed that celiac-superior mesenteric ganglionectomy, alone and in combination with subdiaphragmatic vagal deafferentation, did not alter the anorexia after IP injection of LPS and IL-1 β .⁶¹ Thus, visceral afferent nerves are not necessary for the anorectic effects of IP injected LPS and IL-1 β .

Humoral mediation by cholecystokinin

Cytokine-induced suppression of food intake could result from an enhancement of direct controls of meals

size.⁶² Cytokines induce the production and release of hormones such as cholecystokinin (CCK) (Fig. 1). Intravenous injection of IL-1 β increased plasma CCK^{44,63} and enhanced the CCK-induced increase in afferent vagal activity.⁶⁴ Furthermore, the increase in afferent vagal activity by IL-1 β was significantly reduced in animals pretreated with a CCK $_A$ -receptor antagonist. In one study,⁶⁵ pretreatment with the CCK $_A$ -receptor antagonist L364,718 partially blocked the decrease in food intake and emptying of gastric contents induced by IL-1 β ; this suggests that IL-1-induced anorexia may also be mediated, in part, by peripheral CCK $_A$ -receptors.

It should be noted, however, that peripherally administered cytokines and CCK have different effects on meal patterns, and that the satiating effect of CCK, unlike that of IL-1 β , is attenuated by subdiaphragmatic vagal deafferentation.^{61,66} Therefore, although CCK may contribute to the feeding-suppressive effects of IL-1, it is not the only mediator. It is unknown whether other satiating gut peptides play a role in cytokine-induced suppression of food intake.

Humoral mediation by leptin

Interleukins and TNF α may also reduce food intake by releasing the OB protein (leptin) from adipose tissue (Fig. 1). IL-1 β and TNF α markedly increase leptin expression in adipocytes.⁶⁷⁻⁶⁹ At least for TNF α this seems to be a post-translational effect.⁶⁷ A close correlation between cytokine-induced leptin expression⁶⁸ or serum leptin levels⁶⁹ and food intake reduction has been observed. Findings of an increased and decreased responsiveness to the anorectic effect of injection of LPS in leptin-deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) mice, respectively,⁷⁰ suggest that leptin is not essential for, but contributes to LPS-induced anorexia in this species. We could not detect a differential effect of IP injected IL-1 β and LPS on food intake in lean (*Fa/f*) and obese (*fa/fa*) Zucker rats, which have only some functional leptin receptors. At 3 h after IP IL-1 β (2 μ g/kg) injection, obese and lean rats ate 63% and 64% of vehicle-injected control rats' intakes. Food intake at 12 h after IP LPS (100 μ g/kg) injection was 69% and 77% of control intakes for obese and lean Zucker rats, respectively (Lugarini et al., unpublished). In contrast, the anorectic effect of systemic TNF α was enhanced in obese Zucker rats in a previous study by others.⁷¹ Together, these results suggest that IP injected IL-1 β and TNF α reduce food intake through activation of different mechanisms. Leptin and leptin receptors may be somehow involved, but are not necessary for the anorexia induced by systemic IL-1 β and TNF α .

Central action of circulating cytokines

IL-1 β and TNF α circulating in the blood may reduce food intake by acting at CNS sites directly (Fig. 1). It has

been shown that cytokines can reach CNS cytokine receptors through active or passive transport mechanisms^{72,73} or through circumventricular organs, or they may act through cytokine receptors on the surface of endothelial cells of the brain vasculature and trigger the release of subsequent messengers, such as eicosanoids.^{74,75} Mediators from perivascular microglia may also be involved.⁷⁶ Moreover, cytokines may promote their own uptake into the brain because they increase blood-brain barrier permeability.⁷⁷

The anteroventral region of the third ventricle (AV3V) that includes the organum vasculosum laminae terminalis has been suggested to be a primary site of IL-1 action in the brain, because electrolytic lesions of the AV3V in various animal species decrease the febrile and plasma ACTH responses to systemic administration of IL-1 β .⁷⁸ Moreover, neurons in the AV3V region that send axons to hypothalamic nuclei are responsive to systemically administered IL-1 β ,⁷⁹ but so far the possible role of the AV3V and its hypothalamic projections in the anorectic effect of cytokines has not been critically examined.

The area postrema (AP) is another site where cytokines might gain access to brain circuitries involved in feeding control. Yet, lesions of the AP and the adjacent part of the nucleus of the solitary tract (AP/NST) did not change the anorectic effects of IP administered IL-1 β 26 or TNF α .²⁴ These findings argue against a crucial role of the AP in systemic cytokine-induced anorexia.

The temporal and spatial patterns of *c-fos* mRNA appearance in response to intravenous (IV) injection of IL-1 β support the view that blood-brain barrier endothelial cells play a role in the effects of circulating cytokines on CNS neurons.⁸⁰ The cytokine-induced production and release of eicosanoids by endothelial cells⁸¹ is interesting because the anorectic effects of IL-1 β and LPS have been shown to be attenuated by inhibitors of eicosanoid synthesis.³⁷ NO also plays an important role in endothelial cell function during infections,⁸² but whether NO is involved in the anorexia during immune stimulation is unknown.

Central cytokines

In addition to a central action of systemically produced interleukins and TNF α , *de novo* CNS cytokine synthesis, which has been described in various brain areas relevant to feeding control, may be involved in the anorectic effect of peripheral immune stimulation. *In situ* hybridization, immunohistochemistry, RNase protection and reverse transcriptase PCR studies show that IL-1 β and TNF α mRNA, protein and their receptors are present in several brain regions, including the hypothalamus, and are induced by systemic immune stimulation.⁸³⁻⁸⁷ The situation is complicated, however, because peripheral

immune stimulation can affect brain cytokine and cytokine receptor expression differentially, i.e. while local IL-1 synthesis is increased, IL-1 receptor expression may be decreased by peripheral LPS.⁸⁸ Moreover, in a recent study, IP injection of subseptic doses of LPS induced IL-1 β and TNF α mRNA expression only in the choroid plexus, the circumventricular organs and the meninges.⁸⁹ Thus, the role of CNS cytokine or cytokine receptor synthesis in the anorectic response to peripheral immune stimulation is uncertain.

Some studies found enhanced anorectic effects of ICV administered IL-1 β or IL-1 β plus TNF α in obese (*fa/fa*) Zucker rats.⁹⁰ This enhanced sensitivity did not depend on changes in the brain IL-1 β system at the mRNA level.⁹⁰ The response to ICV IL-6 was also different, whereas ICV TNF α had the same effect on food intake in obese and lean Zucker rats. The reasons for this differential responsiveness are unknown. Obese and lean rat brains may have distinct binding, degrading capacity, clearance and/or uptake for different cytokines.

CENTRAL NEUROCHEMICAL MEDIATION

Independent of the peripheral-to-central communication and the involvement of central cytokines, the anorexia induced by interleukins and TNF α must ultimately result from effects on the CNS mechanisms that control food intake. Some data suggest that cytokines act directly on hypothalamic neurons that are implicated in the control of food intake. Direct application of IL-1 β and TNF α excited the so-called glucoreceptor neurons in the ventromedial hypothalamus (VMH) and suppressed the activity of the glucosensitive neurons in the lateral hypothalamus (see ref. 22). IL-1 β may also affect neuronal excitability through an inhibition of voltage-gated Ca²⁺ channels, an effect that has been shown in hippocampal and hypothalamic neurons.^{91,92}

Increases in serotonergic activity or stimulation of postsynaptic serotonergic receptors decreases and antagonism of serotonin (5-HT) increases food intake (see ref. 93 for review). It is possible that central 5-HT contributes to IL- β -induced anorexia because both centrally and peripherally administered IL-1 β activate central serotonergic activity^{94,95} and because the effect of IL-1 β on 5-HT turnover can be blocked by prior administration of IL-1Ra.⁹⁴ In our hands, several compounds that reduce 5-HT synthesis or transmission (p-chlorophenylalanine, metergoline, and 8-OH-DPAT) were able to block or attenuate the anorectic effect of IP LPS administration (Hrupka et al., unpublished). Injection of the 5-HT receptor antagonist mianserin into the VMH also attenuated the food intake reduction in tumor-bearing rats,⁹⁶ indicating that central 5-HT may be involved in tumor anorexia. The effect of central 5-HT antagonism on the

feeding suppressive effects of peripherally administered cytokines remains to be tested.

While the main role of corticotropin releasing factor (CRF) is to stimulate secretion of ACTH and other proopiomelanocortin products from the pituitary gland, it also reduces food intake.⁹⁷ Peripheral injection of IL-1 β increases hypothalamic CRF mRNA⁹⁸ and IL-1 β -induced anorexia is attenuated by ICV administration of a CRF antagonist.⁹⁷ Thus, CRF is apparently involved in the neurochemical mediation of IL-1 β -induced anorexia.

Histamine is also implicated in the neurochemical control of food intake.⁹⁹ Intraperitoneal IL-1 β injection increased hypothalamic histamine turnover and the activity of histidine decarboxylase and histamine-N-methyltransferase. In addition, depletion of neuronal histamine induced by IP injection of alpha-fluoromethylhistidine attenuated the suppressive effect of IL-1 β on food intake,¹⁰⁰ suggesting that stimulation of a histaminergic mechanism contributes to IL-1 β -induced anorexia.

Alpha-melanocyte stimulating hormone (α MSH) is another important central inhibitor of food intake (reviewed by Tritos and Maratos-Flier in this issue) that may be involved in the production of anorexia by cytokines. Intracerebroventricular administration of α MSH enhanced LPS-induced anorexia in rats,²⁵ whereas administration of the MC3/MC4 receptor antagonist SHU9119 attenuated it.

Neuropeptide Y (NPY) is the most potent known stimulator of feeding behavior¹⁰¹ (see review by Gehlert in this issue). Intracerebroventricularly infused IL-1 β has been shown to cause a small (18%), but significant, reduction in hypothalamic NPY mRNA levels,¹⁰² and ICV injections of NPY can prevent or reverse ICV IL-1 β -induced anorexia.¹⁰³ Anorectic tumor-bearing rats have decreased PVN NPY levels, increased ARC NPY levels,¹⁰⁴ decreased NPY release, and eat less than controls after exogenous ICV NPY administration.¹⁰⁵ These findings suggest an antagonistic interaction between IL-1 β and NPY, and that the feeding suppressive effects of IL-1 β and tumors are in part due to a modulation of NPYergic mechanisms.

INTERLEUKINS AND TNF α IN NORMAL FEEDING AND BODY WEIGHT REGULATION

In addition to orchestrating the immune response during diseases and, as part of this, inhibiting food intake, cytokines might also play a role in the control of normal feeding. Substantial amounts of cytokines are produced in the gut,¹⁰⁶ and food intake represents a high intestinal antigen exposure requiring host defense. Besides local immune activation, this defense may include a coordinated systemic immune response. A high-protein meal did not affect the *in vitro* production or plasma levels of IL-1 β and TNF α , but it decreased the plasma level of

interferon- γ .¹⁰⁷ It also increased the numbers of neutrophils in the blood and decreased monocyte and lymphocyte counts, presumably reflecting emigration of these cells into extravascular lymphoid tissues. Very likely this results in an enhanced local production of proinflammatory cytokines, which could well be involved in limiting the antigenic 'impact', i.e. in inhibiting further ingestion, as part of their defense function. A role of IL-1 β in normal food intake control is also suggested by recent findings of an increase in IL-1 β mRNA expression in rat liver and hypothalamus in response to cafeteria diet feeding.¹⁰⁸ Cafeteria diet feeding also decreased the IL-1R AcP levels in liver and brain stem, suggesting that eating has profound modulating effects on the IL-1 system.

TNF α production in adipose tissue is increased in obese humans^{109,110} and rodents.^{110,111} Adipose tissue TNF α activity increased in mature animals in relation to adipose cell size,¹¹² and with a high-fat diet.¹¹³ Adiposity is also associated with increased basal plasma levels of TNF α .¹¹⁴ TNF α inhibits lipoprotein lipase activity, increases lipolysis in adipose tissue,¹¹⁵ and decreases insulin receptor tyrosine kinase activity. TNF α mRNA expression in adipose tissue is related to the degree of hyperinsulinemia.¹⁰⁹ It is proposed that reduced insulin receptor tyrosine kinase activity by TNF α causes insulin resistance and type II diabetes in obesity.¹¹⁴ Genetically TNF α deficient mice had significantly improved insulin sensitivity in both diet-induced obesity and in the *ob/ob* model of obesity.¹¹⁷ Finally, we recently observed that rats genetically deficient in TNF α and TNF β or in any one of the two TNF receptors ate consistently more than wild-type controls. Moreover, on a high-fat diet, the TNF α and the TNF receptor 2-deficient mice gained substantially more weight than on normal lab chow, whereas the wild-type controls did not (Hrupka et al, unpublished). Although still preliminary, these results might reflect a role of endogenous TNF α in the long-term regulation of food intake and body weight. Interestingly, marked circadian variations of bioactive TNF α with the highest levels at light onset, when feeding is usually low, have been found in rat hypothalamus, hippocampus, and cerebral cortex.¹¹⁸

SUMMARY AND IMPLICATIONS

It is clear that cytokines, in particular interleukins and TNF α , play a major role in the anorexia during many diseases. The mechanisms of cytokine-induced anorexia are not yet fully understood, but are presumably based on interactions between various cytokines and in part between cytokines and other humoral mediators (e.g. leptin and CCK). Moreover, as cytokines are mainly produced in the periphery, whereas anorexia results from a

modulation of the central neurochemical systems that control food intake, peripherally produced cytokines may inhibit feeding indirectly through pathways activated by their peripheral actions. This complex network is even more complicated because the interactions between the immune and nervous systems are bidirectional.^{119,120}

To counteract the anorexia during disease therapeutically, it is necessary to understand its mechanisms. So far, the anorectic effects of cytokines were mainly investigated in animals fed standard laboratory chow. A selective suppression of protein intake by cytokines was observed in a macronutrient choice situation.²⁹ Whether a selective increase in fat and/or carbohydrate content of the diet might prevent the anorexia during disease is questionable, however, because the selection behavior in a nutrient choice situation is of limited predictive value for energy intake in general. In our hands, LPS reduced food intake similarly in rats adapted to equienergetic high fat and high carbohydrate diets.²

The attenuation of IL-1-induced anorexia by inhibitors of eicosanoid synthesis may open the possibility of a nutritional intervention. ω -3 polyunsaturated fatty acids influence eicosanoid production and may act as potential immunosuppressants (e.g. ref. 121). Feeding rats a diet high in fish oil in fact attenuated the anorectic effects of IL-1 β ¹²² and TNF- α .¹²³ Yet, a dietary intervention with polyunsaturated fatty acids in cytokine-induced anorexia will probably be difficult because different effects of polyunsaturated fatty acids on cytokine production are observed dependent on the experimental conditions (see ref. 22).

Attempts to block the anorexia during disease by targeting specific cytokines have yielded mixed results. This is not surprising given the complex interactions and mechanisms of action described above. The difficulty in determining the right time of application and the appropriate dose of a potential cytokine antagonist probably also contribute to some of the negative results. Yet, interleukins and TNF α ultimately suppress feeding by modulating several neurochemical systems that control food intake. Blocking this central neurochemical mediation may be the most promising approach to counteract cytokine-induced anorexia therapeutically because it is presumably the most efficient strategy and does not carry the risk of antagonizing potentially beneficial peripheral effects of cytokines, e.g. during severe cancer anorexia.

Finally, increasing evidence indicates that interleukins and TNF α play a role in the control of adiposity and energy balance, and a detailed analysis of this possibility appears to be a promising area for future research.

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Anticytokine Approaches to the Treatment of Anorexia and Cachexia

Patrick A. J. Haslett

Proinflammatory cytokines such as tumor necrosis factor- α (TNF- α) have a prominent role in the pathogenesis of anorexia and cachexia of chronic disease. Pentoxifylline and thalidomide are inhibitors of TNF- α that have been tried as rational therapeutic interventions in cachexia. Preliminary studies with pentoxifylline have not shown efficacy in reversing weight loss, despite evidence of TNF- α inhibition. In contrast, the administration of thalidomide to patients with human immunodeficiency virus- and/or tuberculosis-associated weight loss has consistently resulted in weight gain. However, the relationship of the metabolic benefits of thalidomide treatment to its complex effects on the immune system is imperfectly understood. Studies of thalidomide, either alone or in combination with other therapies for the treatment of cancer cachexia, are warranted.

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CACHEXIA is a debilitating state of involuntary weight loss complicating chronic malignant, infectious, and inflammatory diseases that contributes significantly to mortality. Anorexia, also a frequent complication of these diseases, is a major contributor to the development of cachexia. However, the pattern of weight loss in cachexia differs from that seen with pure nutrient deprivation. The normal, adaptive response to starvation is to draw on energy-dense lipid stores while sparing protein, resulting in loss of fat and relative preservation of lean tissue. In contrast, cachectic patients experience severe and incapacitating muscle wasting with a relative sparing of adipose tissue. This difference indicates that a maladaptive state of metabolic dysregulation exists in cachexia.

Complex interactions among the nervous, endocrine, and immune systems determine metabolic responses. Abnormalities in all of these systems have been implicated in the multifactorial pathophysiology of cachexia. Although incompletely understood, common mechanisms are likely to underlie the development of cachexia in cancer and human immunodeficiency virus (HIV) disease. Promising therapeutic interventions in one disease state, therefore, have been applied experimentally to others. This is well illustrated by the cross-fertilization of research efforts in the treatment of cachexia complicating cancer and those associated

with advanced HIV disease (acquired immunodeficiency syndrome [AIDS] wasting syndrome).

In general, therapeutic interventions for cachexia have either focused on optimizing nutritional intake or have been empirical, exploiting the effects of drugs such as megestrol acetate, for example, to increase appetite.¹ In general, nutritional interventions have been disappointing, whereas treatment with megestrol acetate has resulted largely in the restoration of adipose body weight.^{2,3}

Ideally, advances in cachexia therapy will result from hypothesis-driven approaches to therapy that attempt to correct specific neuroendocrine or immunologic abnormalities. Examples of endocrine approaches that have anabolic effects used in patients with AIDS and cancer cachexia include the use of recombinant human growth hormone, insulin, and insulin-like growth factor-1 (IGF-1). This article reviews experimental therapies that attempt to modify cytokine responses in human cachexia.

BACKGROUND

In an effort to understand the association between wasting resulting from a chronic infectious disease (experimental trypanosomiasis in a rabbit model) and abnormalities of lipid metabolism, Rouzer and Cerami⁴ first identified a specific circulating mediator of these abnormalities and named it cachectin. Cachectin was subsequently found to be identical to tumor necrosis factor- α (TNF- α), a pro-inflammatory cytokine produced primarily by activated macrophages. Further studies determined that the metabolic and nutritional consequences of overproduction of TNF- α depended on the site of production. Profound and rapidly fatal anorexia accompanied intracerebral production of this cyto-

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Table 1. Summary of Placebo-Controlled Trials of Cytokine Inhibitor Therapy for Anorexia/Cachexia Complicating Cancer, Tuberculosis, or HIV Disease

Study	Drug	n	Disease	Weight Gain	Effect on TNF- α
Goldberg et al ¹⁴	PTX	70	Cancer	No	Not assayed
Tremontana et al ²³	THA	30	TB \pm HIV	Yes	Inhibition
Klausner et al ²⁴	THA	32	TB \pm HIV	Yes	Inhibited in HIV + TB only
Reyes-Teran et al ²⁷	THA	28	HIV	Yes	Not assayed

Abbreviations: PTX, pentoxifylline; THA, thalidomide; TB, tuberculosis.

kine, whereas systemic production resulted in less anorexia and a predominance of metabolic dysregulation.⁵ These early observations illustrate how a complex interplay of metabolic and behavioral factors resulting in cachexia may have a common basis in abnormal cytokine production. In addition, the effects of cytokines are tissue specific and, therefore, may not be reflected accurately in assays of peripheral blood.

In addition to TNF- α , several other cytokines, notably interleukin-1 (IL-1), IL-6, and interferon- α (IFN- α), have now been implicated in the pathogenesis of cachexia associated with human disease. Specific neutralization of these factors with antibodies in animal models of wasting has suggested the validity of an anticytokine approach, while also revealing that no single cytokine is responsible for all of the abnormalities contributing to cachexia. However, because cachexia is a chronic problem in human diseases such as AIDS and cancer, the long-term administration of anti-cytokine antibodies is unlikely to be a practical option. An ideal pharmacologic agent would be orally administered and well tolerated. Pentoxifylline and thalidomide are two agents with cytokine-inhibitory properties currently under investigation as therapies for human cachexia. Controlled studies of these agents in the treatment of cancer and HIV-associated weight loss are listed in Table 1.

PENTOXIFYLLINE

Pentoxifylline, a methylxanthine drug, was originally introduced as a treatment for peripheral

arterial disease based on its potent hemorheological properties. Subsequently, the drug was found to have anti-inflammatory and immune modulatory effects mediated by inhibition of phosphodiesterase.⁶ In vitro, pentoxifylline consistently inhibits TNF- α production by monocytes and T lymphocytes,^{7,8} with mixed reports of its efficacy in inhibiting IL-1 and IL-6.^{8,9} In one study,¹⁰ but not in another,¹¹ the drug was shown to inhibit TNF- α production in human subjects in response to experimentally administered endotoxin. Similarly, the experience with experimental pentoxifylline treatment in diseases where TNF- α is thought to play a pathogenic role has been mixed, with modest clinical effects observed in patients with rheumatoid arthritis but no effect in multiple sclerosis, inflammatory bowel disease, or the Jarisch-Herxheimer reaction.

Recent preliminary investigations in patients with cancer have suggested a possible therapeutic role for pentoxifylline. In 14 cancer patients with TNF- α elevation (which was associated with the adult respiratory distress syndrome or disseminated intravascular coagulation), Lissoni et al¹² reported that intravenous administration of pentoxifylline resulted in a significant diminution in serum levels of this cytokine. A pilot study by Dezube et al¹³ of pentoxifylline administration to cancer patients revealed that TNF- α mRNA expression was reduced in patients who had elevated TNF- α levels at baseline, whereas no reduction was demonstrated in those with normal levels at the start of the study. However, four of five patients with normal baseline TNF- α levels reported an increase in well-being, and two of these experienced a greater than 5% weight gain after 3 weeks of pentoxifylline therapy. This latter observation led the investigators to speculate that pentoxifylline might be inhibiting factors other than TNF- α that are important in the pathogenesis of cancer cachexia. In contrast, a recent double-blind, placebo-controlled study of pentoxifylline therapy for cancer-associated anorexia and/or cachexia did not show an effect on appetite or body weight, although the drug was well tolerated.¹⁴

In HIV disease, TNF- α has been implicated in the regulation of HIV replication, as well as in the pathogenesis of AIDS wasting syndrome. Studies of potential inhibitors of TNF- α in this condition, therefore, have focused on effects on both viral turnover and body weight. Dezube et al^{15,16} have

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published the results of two studies of oral pentoxifylline treatment of HIV-infected patients (400 to 800 mg three times a day) by mouth. Both studies revealed an inhibitory effect on TNF- α , but no effect on body weight or HIV levels. Tolerance of the higher dose regimen was limited by frequent gastrointestinal side effects, notably nausea and vomiting. Similarly, in a pilot study by Landman et al,¹⁷ pentoxifylline treatment of five patients with AIDS wasting syndrome resulted in TNF- α suppression in patients who had elevated TNF- α baseline levels, but it had no effect on body weight.

THALIDOMIDE

Thalidomide was originally introduced as a sedative in the late 1950s. In 1961 the drug was banned from use because of its catastrophic teratogenic effects. The serendipitous recognition in 1964 of the dramatic anti-inflammatory effects of thalidomide in erythema nodosum leprosum (ENL)¹⁸ led to efforts to understand the mechanism of action and potential applications of this drug in other inflammatory diseases. ENL is a debilitating cutaneous and systemic reactional state that complicates the treatment of multibacillary leprosy. This poorly understood syndrome is characterized by inflammatory skin nodules, fevers, weight loss, arthralgias, iritis, and nephritis. ENL is associated with high levels of circulating TNF- α .¹⁹ It was observed that the clinical response of ENL to thalidomide treatment was associated with a precipitous fall in plasma TNF- α levels.²⁰ This finding corresponded with those of *in vitro* studies, which showed that the drug is a specific inhibitor of TNF- α production by human macrophages.²¹

Over the last 30 years, several reports have suggested that the anti-inflammatory benefits of thalidomide may be applicable in a range of disorders, including rheumatoid arthritis, cutaneous lupus erythematosus, and chronic graft-versus-host disease.²²⁻²⁴ More recently, clinical research has focused on the inhibitory effects of this drug on TNF- α , using it both as an experimental treatment for cachexia associated with HIV disease and tuberculosis, and as a tool with which to attempt to understand the role of TNF- α in human disease.

Tramontana et al.²⁵ in a placebo-controlled study, found that thalidomide treatment (300 mg/d for 14 days) resulted in significant weight gain (6% increase in body weight v 2% in the placebo group) in patients with tuberculosis, some

of whom were co-infected with HIV. Thalidomide treatment was associated with suppression of TNF- α production by peripheral blood mononuclear cells, although serum levels of interferon- γ (IFN- γ) were increased. Subsequently, a double-blind, placebo-controlled study confirmed the efficacy of thalidomide (200 mg/d for 21 days) in causing weight gain in patients with HIV-associated wasting, with or without concomitant tuberculosis.²⁶ In the latter study, however, inhibition of TNF- α only was found in the subset of patients with tuberculosis co-infection, whereas patients with uncomplicated HIV infection did not have high TNF- α levels at baseline. In parallel with these findings, plasma titers of HIV declined in response to thalidomide in the tuberculosis co-infected group only. A further placebo-controlled study of thalidomide (400 mg/d for 12 weeks) in patients with AIDS wasting syndrome found weight stabilization in eight of nine thalidomide-treated patients compared with two of nine in the control group.²⁷

In an attempt to elucidate the nature of the weight gain seen in HIV-infected patients in response to thalidomide therapy, and to distinguish a metabolic from an appetite-stimulating effect, a recent study examined the effect of thalidomide treatment (200 mg/d for 14 days) in 12 weight-stabilized HIV-infected patients receiving an isocaloric metabolic diet.²⁸ A mean increase of 3.06% above baseline body weight was observed. This was associated with a mean nitrogen retention of 25.34 g ($P \leq .02$) over the period of treatment, suggesting significant net protein anabolism. Body composition was measured by bioelectric impedance analysis. A trend toward fluid retention during the first week of thalidomide therapy, followed by lean tissue expansion during the second week was observed, although these changes were not statistically significant. Thus, thalidomide treatment caused weight gain independently of an effect on appetite. Of course, this does not exclude the possibility that thalidomide treatment also may have appetite-stimulating effects.

In this latter study, plasma TNF- α levels were not elevated at baseline and were not suppressed by thalidomide treatment. However, plasma levels of soluble IL-2 receptor increased significantly during the treatment phase, suggesting paradoxically, drug-induced immune activation. Another study, which demonstrated the efficacy of thalidomide in

healing HIV-associated oral aphthous ulceration, found drug-induced increases in plasma levels of both TNF- α and HIV.²⁹ These latter findings also are consistent with thalidomide-induced immune activation.

Historically, thalidomide has been quite well tolerated by patients without HIV infection. Other than the notorious teratogenicity of this drug, the most serious toxicity is an axonal peripheral neuropathy, which may be irreversible.³⁰ Constipation is a frequent, relatively minor problem, although this may become a concern in cancer patients receiving opiate palliation. In contrast, toxicities are common in patients with HIV disease treated with thalidomide, with rashes and fevers affecting approximately one third of patients. The latter toxicities appear to be more frequent in patients with more advanced HIV disease.³¹

There are no published reports in which thalidomide has been used specifically as therapy for cancer cachexia. However, the discovery of another property of thalidomide, the inhibition of angiogenesis,³² has led to investigational studies of this agent as an antiangiogenesis agent for various tumors.^{33,34}

OTHER DRUGS

There have been few other attempts to pharmacologically modify immune or inflammatory responses that may contribute to the pathogenesis of cachexia. A recent pilot study by McMillan et al.³⁵ of combined megestrol acetate and ibuprofen therapy for patients with gastrointestinal cancer showed significant weight gain and a reduction in C-reactive protein levels. Megestrol acetate was demonstrated to inhibit the production of TNF- α , IL-1, and IL-6 by mitogen-stimulated peripheral blood mononuclear cells in vitro,³⁶ suggesting that this agent may also act in part by modulating cytokine responses. Suramin, an agent used in the therapy of African trypanosomiasis, was found to ameliorate experimental cancer cachexia in a mouse model and to inhibit binding of IL-6 to its receptor.³⁷ However, the nephrotoxicity of suramin would limit its application in humans.

CONCLUSIONS

In published reports, pentoxifylline and thalidomide are the only two pharmacologic agents that have been administered as cytokine inhibitors for the experimental treatment of AIDS or cancer

cachexia. Both drugs are inhibitors of TNF- α . However, these agents also are likely to have additional effects on immune and inflammatory responses. In general, the few trials of pentoxifylline therapy in cancer and AIDS cachexia have been disappointing, achieving no increase in body weight despite demonstrations of TNF- α inhibition. In contrast, studies with thalidomide have uniformly shown weight gain in patients with HIV and/or tuberculosis, with inhibition of TNF- α and evidence of immune stimulation demonstrated in some situations. There are no published reports of thalidomide therapy for cancer cachexia. The experience with chronic infectious disease suggests that this agent should be studied in patients with cancer cachexia, both as a single agent and in combination with an appetite stimulant such as megestrol acetate. Additionally, it is worth noting that the antiemetic properties of thalidomide, which led to its widespread and disastrous use in early pregnancy, may be of additional benefit in cancer patients with chronic nausea.

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MINI-REVIEW

Mechanisms of alcoholic liver injury

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SW French. Mechanisms of alcoholic liver injury. *Can J Gastroenterol* 2000;14(4):327-332. There have been numerous recent advances in the understanding of the mechanisms of alcoholic liver disease pathogenesis. Endotoxin-induced Kupffer cell activation plays a role in cytokine-mediated inflammatory changes in the liver, and this can be blocked by a diet high in saturated fat, by a diet containing lactobacillus, which does not produce endotoxin, by neomycin antibiotic sterilization of the gut, by eliminating Kupffer cells, or by removing tumour necrosis factor- α with antibody or by using tumour necrosis factor- α knockout mice. The fatty liver component is mainly the result of the nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide redox shift to the reduced state by ethanol oxidation generation of reduced nicotinamide adenine dinucleotide, although this too can be blocked by a diet high in saturated fat. Hepatocytic enlargement occurs due to ethanol-induced inhibition of the ubiquitin-proteasome pathway of cytoplasmic protein degradation and the retention of oxidized proteins in hepatocytes. The liver is scarred by stellate cells that have been activated by inflammatory cytokines and growth factors produced by activated Kupffer cells, and by bile ductule metaplasia. Mallory bodies and balloon cell degeneration develop through the ethanol-induced oxidative stress-protein kinase activation pathway, inhibition of phosphatase activity and inhibition of the ubiquitin-proteasome pathway.

Key Words: Cytokines; Ethanol; Growth factors; Kupffer cells; Stellate cells

Mécanismes d'apparition des lésions hépatiques dues à l'alcool

RÉSUMÉ : De nombreux progrès ont été réalisés récemment dans la compréhension des mécanismes de la pathogénèse du foie alcoolique. L'activation des cellules de Kupffer, d'origine endotoxique, joue un rôle dans les changements inflammatoires médiés par les cytokines, qui se produisent dans le foie. Le processus peut être inhibé par un régime riche en graisses saturées ou contenant des *Lactobacillus*, qui ne produisent pas d'endotoxine, la stérilisation de l'intestin à la néomycine, l'élimination des cellules de Kupffer, ou encore par l'élimination du facteur- α de nécrose des tumeurs à l'aide d'anticorps ou l'utilisation du facteur- α de nécrose des tumeurs inactivé de souris. La stéatose hépatique résulte surtout du passage de l'oxydoréduction du nicotinamide adénine dinucléotide/nicotinamide adénine dinucléotide réduit à l'état réduit par la production de nicotinamide adénine dinucléotide réduit par l'oxydation de l'éthanol, bien que le processus puisse, lui aussi, être bloqué par un régime riche en graisses saturées. L'augmentation de volume des hépatocytes est attribuable à l'inhibition de la chaîne ubiquitine-protéasome de la dégradation des protéines cytoplasmiques et à la rétention des protéines oxydées dans les hépatocytes. L'intégrité du foie est attaquée par les cellules étoilées activées par les cytokines inflammatoires et les facteurs de croissance produits par l'activation des cellules de Kupffer, ainsi que par la métaplasie des voies biliaires. L'activation de la protéine-kinase associée au stress oxydatif de l'éthanol, l'inhibition de l'activité de la phosphatase et l'inhibition de la chaîne ubiquitine-protéasome sont responsables de la dégénérescence des cellules ballonisées et de la production des corps de Mallory.

Progress in understanding how ethanol causes liver damage has been made possible through the use of rodent models of alcoholic liver disease (ALD). Isolation of liver cellular constituents in culture and the use of mouse knockout models have further focused investigations of the mechanisms involved. The roles of inflammatory cytokines and chemokines, as well as of growth factors have been further

defined (Figure 1). Progress in understanding the role of metabolic changes and consequences of oxidant stress has revealed new concepts regarding liver injury and subsequent fibrosis and cirrhosis in animal models. The mechanisms of hepatocellular swelling, cytochrome aggregation (ie, Mallory bodies [MBs]) and apoptosis are better understood. This progress is the subject of the present review.

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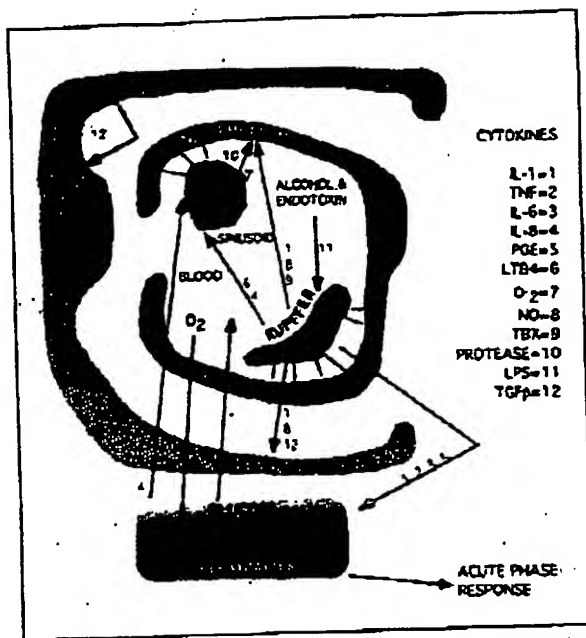


Figure 1) Schematic representation of the four types of cells involved in the focal sinusoidal inflammatory response to Kupffer cell activation in alcoholic liver disease. Cell to cell communications result from various secretory products and cell receptors. Cytokines, proteases, free radicals, eicosanoids and endotoxin are involved in paracrine and autocrine stimulated responses (arrows). IL Interleukin; LPS Lipopolysaccharide; LTB4 Leukotriene B4; NO Nitric oxide; PGE Prostaglandin E; PMN Polymorphonuclear leukocytes; TBX T-box; TGFβ Transforming growth factor-beta; TNF Tumour necrosis factor

The role of the Kupffer cell as the primary target of ethanol liver damage has been summarized in depth by Lands (1), and was first revealed with the observation by Nanji et al (2) that endotoxin levels were elevated in the blood of rats fed ethanol intragastrically for one to two months. They noted a significant correlation between endotoxin (lipopolysaccharide [LPS]) levels and the severity of the liver pathology (ALD) that induced fatty change and liver cell necrosis. Nanji et al (3) showed subsequently, using the same model, that replacing gut bacteria with lactobacillus, which do not produce endotoxemia, prevented the development of ALD pathology. This has been confirmed by reducing LPS in the blood by feeding the rats neomycin to sterilize the gut (4). The significance of this observation became apparent when it was shown, with the use of the same rat model, that the liver pathology caused by ethanol feeding was largely prevented by eliminating the Kupffer cells with gadolinium chloride treatment (5). Because Kupffer cells are activated by LPS to produce a large number of inflammatory cytokines and bioactive, potentially hepatotoxic substances, it was reasonable to suspect this cell as the central mediator of ALD.

One of the candidate cytokines produced by Kupffer cells in response to LPS is tumour necrosis factor- α (TNF α). This cytokine is elevated in the blood of patients with ALD,

as is LPS (6,7), and probably accounts for the anorexia that these patients experience. Nanji et al (8) reported that livers from rats with experimental ALD showed increased TNF α mRNA expression. Kupffer cells isolated from the rat model showed increased gene expression of TNF α , interleukin (IL) -6 and transforming growth factor-beta (TGF β) (9). In fact, antibodies to TNF α attenuated the ALD pathology (10,11), further substantiating that Kupffer cell overproduction of TNF α is an important step in the development of ALD.

Of course, Kupffer cell activation by LPS and ethanol leads to increased secretion of cytokines IL-1, -6 and -8, TGF β and the chemokines chemokine cytokine-induced neutrophil chemoattractant, macrophage inflammatory protein (MIP), monocyte chemoattractant protein-1 (MCP-1), platelet-activating factor and insulin-like growth factor 1 (IGF-1), through the activation of nuclear factor kappa B (NF κ B) transcription (1). Oxidative stress activates NF κ B, leading to the induction of inducible nitric oxide synthase and nitric oxide generation (1). Through the CD14, receptor LPS activates phospholipase C and protein kinase C (PKC). In the Kupffer cell (12), increases cytosolic calcium and increases reduced nicotinamide adenine dinucleotide phosphate oxidase generation of superoxide (1). The LPS-CD14 response is augmented by the liver cell release of LPS-binding protein, an acute phase response protein stimulated by IL-6 produced by the Kupffer cell. Cyclo-oxygenase-2 is induced in the Kupffer cells, which leads to an increase in thromboxane A₂, a potent agonist for vasoconstriction, thrombosis, ischemic necrosis and inflammation in the liver (13). This is augmented by activation of phospholipase A₂ generation of arachidonic acid (13). PKC is also activated by oxidative stress, which activates tyrosine kinase and the mitogen-activated protein (MAP) kinase cascade. Both the increase in cytosolic calcium and the MAP kinase cascade increase phospholipase A₂, which probably accounts for the activation and sensitization of the Kupffer cell to augment further injury caused by further challenges with ethanol. Kupffer cells undergo an acute desensitization after challenge followed by increased sensitivity to rechallenge a few hours later (14).

Kupffer cell-mediated injury is amplified by cytokine-mediated induction of cell adhesion molecules (integrins, ie. intercellular adhesion molecule, vascular cell adhesion molecule, IL-8 and leukotriene B₄). Monocytes, lymphocytes and neutrophils are thus immobilized within the sinusoid. These inflammatory foci generate superoxide and proteases. Myeloperoxidase generates hypochloric acid, leading to focal damage and hepatocellular necrosis.

Active Kupffer cells also release proteases such as urokinase, which may affect neighbouring hepatocytes because urokinase activates hepatocyte growth factor (HGF) to stimulate hepatocellular regeneration.

There are three mitigating circumstances where ethanol ingestion may not activate Kupffer cells. For instance, a high fat diet is required if ethanol is to induce ALD pathology by activating Kupffer cells (15), ie. NF κ B activation, TNF α and MIP-1 mRNA expression. Another modifier is the in-

hibitory action of acetaldehyde. Drugs that block acetaldehyde dehydrogenase and increase the levels of liver tissue acetaldehyde prevent the ALD pathology and Kupffer cell NF κ B activation (16). A third factor is that increased non-heme iron levels in Kupffer cells are critical to ethanol activation of Kupffer cells and TNF α expression (15). The latter may be the mechanism of cirrhosis formation that is observed in the rat ALD model when a small amount of carbonyl iron is added to the diet (17).

Of great interest is the mechanism of liver cell necrosis in ALD. In the rat model as well as in human ALD, apoptosis is clearly increased (18,19). The apoptotic process is short lived (3 to 5 h), so the phenomenon is easy to miss. Because TNF α can induce apoptosis *in vitro* (20), this may be the mechanism in ALD; however, NF κ B activation prevents TNF α -induced apoptosis. One theory is that glutathione depletion in the mitochondria, which results from oxidative stress in hepatocytes, leads to membrane permeability transition change, calcium influx into the mitochondria, mitochondrial swelling and release of cytochrome C. Cytochrome C binds to Apaf-1 to activate caspase 9, which activates caspase 3, triggering a cascade of events that lead to programmed cell death and the breakup of the hepatocytes into small fragments followed by their removal by phagocytosis (20). Others postulate that TNF α signalling involves sphingomyelinase formation of ceramide as a second messenger that triggers the formation of reactive oxygen species, which initiates mitochondrial membrane permeability transition change, which results from the alteration of complex III of respiration (21).

Hepatocellular changes that are probably independent of Kupffer cell activation include fat accumulation, fatty acid metabolic changes, loss of glycogen stores, inhibition of proteolysis, hyperphosphorylation of cyokeratins and the oxidation of lipids, proteins and DNA.

The accumulation of fat in the form of triglyceride is multifactorial in terms of the mechanisms involved. Most likely, fat accumulates mainly due to the decreased beta oxidation of fatty acids by the mitochondria due to the shift in the oxidized form of nicotinamide adenine dinucleotide to reduced nicotinamide adenine dinucleotide ratio (22). Fatty acid metabolism is greatly altered due to the induction of cytochrome P450 (CYP) enzymes, which peroxidize, hydroxylate and epoxidize them to bioactive intermediates (hydroxyeicosatetraenoic acids and epoxyeicosatrienoic acids) (23), and enzymes that metabolize arachidonic acid to bioactive prostaglandins and thromboxanes (13). Lipid peroxidation leads to protein adduct formation, which correlates positively with the severity of liver pathology in the rat and humans (24,25). Intermediates are formed from fatty acid hydroxylation as the result of the induction of cytochromes such as CYP 4A1, CYP 3A1, CYP 2B1 and CYP 1B1 (26-28). The adducts formed with proteins act as neoantigens on the liver cell surface, and antibodies bind to these adducts on the plasma membrane to damage the liver cells, hypothetically, by antibody-assisted lymphotoxicity mechanisms.

Liver cell proteins become damaged through peroxidation by the CYP 2E1-generated free radical formation of peroxyls and alkoxyls, which leads to the accumulation of oxidized proteins in hepatocytes (29). Concomitantly, ethanol feeding leads to a decrease in ubiquitin in hepatocytes (30). Ubiquitin is necessary to prepare the cytosolic proteins for degradation by the proteasome pathway. Compounding this problem in the removal of cytosolic proteins, ethanol feeding leads to inhibition of the proteolytic enzymes in the proteasome (31). Consequently, proteins accumulate in hepatocytes (31), which probably accounts for the 'ballooning' of hepatocytes seen in ALD. Oxidized proteins, in increased amounts, inhibit protein degradation by the proteasomes (32).

Oxidation of mitochondrial DNA is also induced by chronic alcohol ingestion (33). Mitochondrial DNA deletions occur in the liver of alcoholics associated with steatosis (34). Both errors in cytosine loci where wrong nucleotides are incorporated during replication of DNA cause point mutations (34). In addition DNA, deletions are observed in the liver mitochondria of alcoholics (35). These changes are reversible when alcoholic patients stop drinking (36).

The cell origin of collagen in the liver, including the source of scarring and cirrhosis, is the stellate cell, formerly known as the Ito cell or lipocyte. Recent focus on the role of this cell in ALD has revealed that it is activated by fibronectin and cytokines IL-1, TGF β and IL-6. Fibronectin is derived from sinusoidal endothelial cells (37,38). It is increased in the space of Disse early in the course of ethanol feeding (39), followed by an increase in collagen IV (40), followed by capillarization of the sinusoid by laying down basement membrane by the endothelial cells (40). Although the focal activation of stellate cells that is due to focal necrosis occurs early in the course of experimental ALD (one to two months of alcohol feeding), diffuse activation of stellate cells occurs late in the course of ethanol feeding (five to six months of feeding ethanol) (41). Extensive centrilobular perisinusoidal fibrosis due to diffuse stellate cell activation requires polyunsaturated fatty acids in the diet (42-44) and a high fat diet (14).

Activation of stellate cells, either focally or diffusely, probably results from paracrine stimulation by Kupffer cells, endothelium and hepatocytes, which release TGF β IL-1, TGF α , platelet-derived growth factor (PDGF), TNF α and IGF-1 (37,45,46). Acetaldehyde (15) and interferon (45) inhibit stellate cell activation. This may explain why activation of stellate cells *in vivo* takes so long to be induced by ethanol. Activated stellate cells become responsive or hyperresponsive in tissue culture to cytokines and growth factors through paracrine and autocrine mechanisms. These factors include IGF-1, TGF α , TGF β , colony stimulating factor-1, HGF, IL-6, PDGF, epidermal growth factor (EGF), MCP-1, fibronectin and endothelin-1 (37,46). The activated stellate expresses a complex phenotype that includes the following newly acquired functions: proliferation, migration, contraction, collagen and fibronectin synthesis, white blood cell chemotaxis and collagenase secretion. Focal in-

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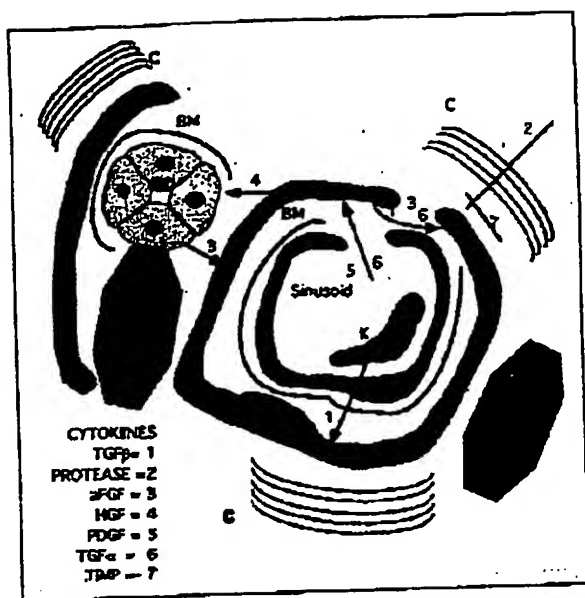


Figure 2) Schematic representation of pericellular fibrosis and bile duct metaplasia induced fibrosis. Cytokines, proteases, growth factors and inhibitors are involved. aFGF Acidic fibroblast growth factor; BD Bile duct; BM Basement membrane; C Collagen; E Endothelium; H Hepatocyte; HGF Hepatocyte growth factor; K Kupffer cell; PDGF Platelet-derived growth factor; S Stellate Cell; TGFα Transforming growth factor; TIMP Tissue inhibitors of matrix metalloproteinases

jury, leading to local scarring, is quickly resolved unless the stimulus is sustained. It is not known which factors perpetuate the scarring process that leads to cirrhosis. Scars that do not lead to septal formation can be completely removed by collagenase over time. Thus, permanent scarring is the result of the balance between collagen synthesis and degradation over time. Collagenase inhibitors (tissue inhibitors of matrix metalloproteinases) may be important in maintaining this balance (47).

Activation of stellate cells leads to the deposit of a great variety of extracellular matrix proteins (37) secreted by the stellate cells. Among these is hyaluronic acid (37), which is elevated in the blood during active liver fibrosis. Hyaluronic acid is normally cleared by hepatic endothelial cells, but in hepatic fibrosis in ALD the endothelial cells fail to clear it (48). The CD44 receptor isoform for hyaluronic acid is expressed by stellate cells in liver injury, and this may be a factor in the migration of stellate cells to the site of injury in the localized fibrosis in ALD (49).

One important property of the activated stellate cell is its enhanced contraction in response to endothelin-1 from endothelial cells (50). Thus, activated stellate cell contraction may explain the damage to hepatocytes that occurs in the centrilobular zone because of the liver hypoxia that develops in rats fed ethanol chronically (51).

One important mechanism by which stellate cell proliferation plays a role in the pathogenesis of alcoholic cirrhosis involves the liver cell bile ductular metaplasia phenomenon (52). In this phenomenon, liver cells undergo a phenotypic

switch to form ductules in the limiting plate of hepatocytes at the periportal zone during the development of cirrhosis (18). The stellate cells located next to the switched hepatocytes proliferate to provide stroma for the newly formed ductules in a process that recapitulates bile duct formation in the fetal liver (53). This is a self-perpetuating interaction that leads to progressive fibrosis and cirrhosis due to growth factors released by the ductules (acidic fibroblast growth factor), which stimulates stellate proliferation, and HGF and stem cell factor released by the stellate cells, which stimulates bile ductule proliferation (54,55). Endothelin-1 (56), TGFβ (57), EGF and IL-6 (58) may also participate in this stimulation-proliferation process of periportal fibrosis (Figure 2).

The role of MB formation in ALD has recently been better defined. The formation of MBs in drug-primed mice fed ethanol intragastrically for seven days provides a model to determine their pathogenesis (59) because of the short interval between starting ethanol ingestion and MB formation. In this model, CYP 2E1 was induced and cytochrome P-450 was increased, whereas the mRNA for CYP 2E1 tended to decrease and cytochrome P-450 mRNA was significantly decreased. The discrepancy between the protein levels and the mRNA expression indicates an inhibition of proteolysis by the proteasome as the mechanism for cytochrome P-450 and CYP 2E1 accumulation. This fits the hypothesis that MBs result from the accumulation of cytochromes that are conformationally altered so as to resist proteolysis, which is inhibited by ethanol ingestion (60). Evidence that the MBs are composed of cytochromes that have undergone profound alterations in their conformation was obtained by infrared spectroscopy of the amide I spectrum of isolated mouse and human MBs (61).

Hyperphosphorylation of MBs (60) likely accounts for their conformational change. Secondary ubiquitination (62) of MBs may add to their resistance to proteolysis. The question of what is the mechanism of hyperphosphorylation of MBs remains. Studies on MB phosphorylation indicate that PKC is the main kinase involved, probably triggered by oxidative stress. Ethanol induces hyperphosphorylation of cytochromes in hepatocytes within 15 mins in primary tissue culture of hepatocytes through a PKC mechanism (63). Okadaic acid increases phosphorylation of liver cytochromes in vivo within 15 mins by inhibiting serine-threonine phosphatases 1 and 2A (60). Hyperphosphorylation was indicated using an antibody to phosphothreonine. Aggregates of cytochromes resulted, and these stained positive for cytochromes, ubiquitin and phosphothreonine in the same manner as do MBs (60). The aggregates were present in 'empty' hepatocytes that failed to stain for cytochromes except where the aggregates were found. The 'empty' cells correspond to the balloon cells containing MBs seen in ALD in humans (64). These acute changes induced by okadaic acid occurred at the same time that NFκB was activated (60), which is a further indication that oxidative stress was involved (65). The hyperphosphorylation of serine residues (66) as well as the involvement of PKC has been corroborated, although other kinases may also be involved (67).

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INFOTRIEVE 3

Treatment of Sepsis

Past and Future Avenues

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Abstract

In recent years, the concept has emerged that the host's inflammatory response contributes substantially to the development of septic shock and organ failure. Experimental observations prompted large scale randomised clinical trials with a variety of agents such as glucocorticoids, ibuprofen, antiendotoxin monoclonal antibodies, antagonists of platelet-activating factor, of bradykinin or of interleukin-1 receptor, and monoclonal anti-tumour necrosis factor (TNF) antibodies or soluble dimeric TNF receptor fusion proteins.

All these major studies of immunomodulators in sepsis have yielded disappointing results despite showing promise during preliminary clinical studies. However, these recent failures do not mean that septic shock will forever remain an insurmountable medical challenge. Many lessons have been learned from these studies, and certain mistakes in their study design will be avoided in the future. Our understanding of the pathophysiology of sepsis and septic shock is increasing markedly; potential new treatment strategies are available and could be explored to improve the outcome of patients with sepsis.

Our understanding of the pathophysiology of sepsis and septic shock has increased markedly over the past few years.^[1-5] Sepsis begins when bacteria cross host barriers, overwhelming host defences, and release toxic bacterial products that activate plasma factors (complement and clotting molecules) and cells of the immune system (monocytes/macrophages, polymorphonuclear cells and lymphocytes). Cell receptors for toxic microbial products and serum factors that enhance host responses to bacterial products have been characterised. Upon activation by bacterial products, host cells release a complex array of mediators, including cytokines. Cytokines are autocrine and paracrine molecules that act locally at their site of production and serve to orchestrate the cellular and humoral host responses. By influencing coagula-

tion and leucocyte transmigration, and by activating professional phagocytes, cytokines assist the host to contain a local infection.

However, in septic shock, this process is out of control. The synthesis of pro-inflammatory molecules, especially tumour necrosis factor (TNF) and interleukin (IL)-1, upregulates the expression of adhesion molecules on endothelial cells, promoting the accumulation of activated polymorphonuclear cells. This leads to further cytokine production and release of toxic molecules from polymorphonuclear cells, resulting in endothelial necrosis and vascular permeability. Septic shock is a manifestation of a dysregulated inflammatory response during which the counter-regulatory systems, including anti-inflammatory cytokines (IL-4, IL-10, IL-13, transforming growth factor β , granulocyte

colony-stimulating factor) soluble TNF receptors, IL-1 receptor antagonist and glucocorticoids, are overwhelmed. The pro-inflammatory response affects vascular permeability and resistance and cardiac function, and induces many metabolic derangements often leading to multiorgan failure and death.

1. Where Are We Now?

In recent years, the concept has thus emerged that the host's inflammatory response contributes substantially to the development of shock and organ failure. Although many experimental studies have focused on Gram-negative septic shock, we now know that endotoxin is not the universal trigger of septic shock that it was once believed to be. Gram-positive bacteria also can cause a clinical syndrome that is similar to Gram-negative septic shock, but the cellular and molecular mechanisms of Gram-positive shock have not been fully elucidated. Systemic fungal infections can also produce sepsis and septic shock, yet the pathophysiology of fungal sepsis remains largely unknown.

The current strategies of adjunctive therapy for sepsis are mainly derived from observations made in animal models. Promising experimental results prompted large scale randomised clinical trials with a variety of agents such as antiendotoxin (antilipid A) monoclonal antibodies,^[6-9] glucocorticoids^[10,11] or ibuprofen^[12] for nonspecific down-regulation of inflammation, antagonists of platelet activating factor,^[13,14] of bradykinin^[15] or of IL-1 receptor,^[16,17] and monoclonal anti-TNF antibodies^[18-21] or soluble dimeric TNF receptor fusion proteins.^[22,23] Unfortunately, despite some promising results during preliminary trials, all the major clinical studies of immunomodulators in sepsis have yielded disappointing results (for discussion see, for example, Bone,^[24] Abraham and Raffin^[25] and Zeni et al.^[26]). A phase III study of a p55 TNF receptor IgG1 fusion protein was recently terminated after inclusion of 1342 patients. The preliminary results showed no difference in the 28-day mortality rates between the patients receiving the fusion protein and placebo, respectively. Analysis

of subgroups showed that no subgroup showed convincing benefit (E. Abraham, personal communication).

There are several explanations for the discordant results observed between animal models and in clinical trials. Major differences exist between animal models of sepsis and clinical septic shock. In animal models, the cascade of events is perfectly synchronised and follows a predictable and brief course usually ranging from a few hours to a few days. The initial stimulus is usually given as a single, titrated dose, via the same route, to healthy animals with the same genetic background. The infections or toxic challenges are accompanied by the release of a transient and usually single burst of each cytokine. Experimental protocols aimed at blocking a single cytokine cascade are thus relatively straightforward. In animal models, cytokine blockade is efficient only when performed prophylactically or very early after challenge, which is obviously not feasible in humans.

In contrast, the sequence of events leading to septic shock in humans is much more complex, asynchronised, and extends over a prolonged period of time, usually many days. Therefore, immunological interventions have to work in a very complex environment in which opposing pro- and anti-inflammatory forces are in action. In fact, in late shock, it might even be that the pro-inflammatory burst is over and that the patient is in a state of hyporesponsiveness which would require stimulation rather than inhibition.

Furthermore, the design of the clinical trials themselves raises several unresolved issues, such as the criteria used for the selection of the endpoints (for example, mortality versus morbidity, all-cause death versus death due to septic shock only, 28-day mortality versus early mortality), and the existence of confounding factors (for example, decision not to resuscitate, inadequacy of treatment, underlying diseases).

However, perhaps the major issue is the selection of patients. Indeed, in all recent clinical trials, the selection of patients was based on the sepsis criteria proposed by Bone et al.,^[27] a method that

has been criticised.^[28] The concept of sepsis syndrome relies on the belief that the pathophysiology of sepsis and organ dysfunction is similar whatever the microbial aetiology and primary focus of infection may be. However, there are 2 major problems with this concept. First, the systemic manifestations of infection that can be readily recorded are highly nonspecific and can also occur with many noninfectious illnesses. Secondly, the assumption that the pathophysiology of sepsis is similar for all micro-organisms and primary foci of infections is doubtful. The concept of sepsis syndrome became very popular because it allows the inclusion of a substantial number of intensive care unit patients in clinical trials, allowing the performance of the large-scale randomised trials that are required for statistical reasons. However, the power of these studies was lower than expected because the selection of patients was based on the nonspecific criteria of the sepsis syndrome.

Although the clinical studies performed so far have not allowed the conclusion that, for instance, TNF or IL-1 blockade is effective in patients with sepsis or septic shock, we do not yet really know whether these agents have some protective efficacy or not. Future clinical trials in this field should have carefully designed entry criteria no longer based on the single concept of sepsis syndrome. These trials should also select more homogenous patient populations, such as for example those with intra-abdominal infections, nosocomial pneumonia or fulminant meningococcaemia. Timing of intervention is also crucial. In fact, in the recently completed anti-TNF multicentre studies,^[23,29,30] only a small proportion of the patients had detectable circulating TNF levels at entry, raising the possibility that treatment might have been administered too late, after peak TNF release in the blood. This stresses the need for laboratory tests to help clinicians identify at the bedside those patients who are likely to benefit from the experimental drug.

2. Where Do We Go?

The fact that all recent clinical trials of anti-inflammatory agents have yielded disappointing

results does not mean that septic shock will forever remain an insurmountable medical challenge. Many lessons have been learned from previous studies and the failure of the current approaches should stimulate researchers to find new treatment modalities. As shown in table I, many strategies are available to interrupt the cascade of events that occurs during septic shock. Although some of these treatment approaches are still at a preclinical stage, others are, or will soon be, in clinical development. It is beyond the scope of this article to review all these treatment options, and therefore only some will be considered.

Despite the lack of efficacy of antiendotoxin antibodies, one approach worth pursuing is to block the ability of microbial agents to activate target cells. Candidate molecules are lipid A antagonists,^[31-34] acyloxyacyl hydrolase,^[34] bactericidal/permeability-increasing protein,^[35] cationic antimicrobial proteins (CAP18 and 37),^[36] antilipopopolysaccharide factor^[37] and high density lipoproteins (HDL). Lipoproteins are natural antagonists of endotoxin (lipopolysaccharide; LPS). Recently, it was found that LPS binding protein (LBP) and soluble CD14 also catalyse the transfer of LPS to HDL.^[38,39] Administration of lipoproteins decreases cytokine production *in vitro* and *in vivo* and protects animals from endotoxic shock.^[40-44] A phase II study of reconstituted HDL for the treatment of peritonitis will soon be initiated in Europe.

Another treatment option is to prevent the activation of responsive cells by microbial products. LBP and CD14 (both its membrane-bound and soluble forms) are 2 critical components of the activation of host cells by LPS. Recent investigations have shown that CD14 is also involved in the recognition of many other micro-organisms, including Gram-positive bacteria, mycobacteria and yeasts, and is thus an integral component of innate immunity.^[45] In animal models, blockade of LBP has been shown to prevent death from shock induced by endotoxin or by Gram-negative bacteria,^[46] and transgenic mice overexpressing human CD14 were found to be hypersensitive to LPS.^[47] Recent experiments with LBP- or CD14-knockout

Table 1. Present and future strategies for the treatment of patients with sepsis**1. Blocking the release or the action of microbial products**

Neutralisation of lipopolysaccharide

- lipid A antagonists
- acyloxyacyl hydrolase
- antilipopolysaccharide factor
- bactericidal/permeability-increasing protein
- cationic antimicrobial proteins
- reconstituted high density lipoprotein

Neutralisation of bacterial toxins or microbial cell walls with inhibitors or antibodies

2. Preventing the activation of responsive cells

Blocking lipopolysaccharide-binding protein or CD14

Interfering with intracellular signalling cascades

- tyrosine kinases
- mitogen-activated protein kinase superfamily
- lipid mediators (phospholipases, diacylglycerols, phosphatidylinositol 3,4,5-trisphosphate, protein kinase C, sphingomyelin, ceramide)

Inhibiting the action of transcription factors (nuclear factor- κ B, activator protein-1)**3. Inhibiting secondary mediators**

Cytokines

- inhibition of cytokine synthesis and release (steroids, interleukin-10, phosphodiesterase inhibitors, thalidomide, CNI-1493, inhibitors of tumour necrosis factor converting enzyme)
- neutralisation of cytokine activity (monoclonal antibodies, soluble receptors, receptor antagonists)

Nitric oxide

- specific and nonspecific inhibitors of inducible nitric oxide synthase

Lipid mediators

- platelet-activating factor antagonists
- blockade of the cyclo-oxygenase-dependent and the lipoxygenase-dependent pathways

4. Blocking the activation of humoral pathways

Inhibition of coagulation (antithrombin III, inhibition of tissue factor pathway)

Inhibition of complement

Inhibition of kinin (bradykinin antagonists)

5. Preventing the activation of target cells

Neutralisation of adhesion molecules (selectins, integrins)

6. Immunostimulation

- Interferon- γ , granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, interleukin-12, interleukin-18

The elucidation of the signalling pathways involved in gene expression after the activation of the cell membrane receptor by LPS or other microbial agents has been an area of intensive research in recent years. Several signalling cascades have been identified, including the mitogen-activated protein (MAP) kinase superfamily.^[50] Each component of these pathways is a potential target for drug development. Small inhibitors of these signalling molecules should soon be available for testing in experimental animal models of sepsis. Further downstream targets are the transcription factors nuclear factor (NF)- κ B and activator protein (AP)-1, which play a critical role in the expression of many pro-inflammatory mediators and acute phase proteins.

The inhibition of secondary mediators, such as the cytokines, nitric oxide or the lipid mediators, has been extensively studied in the last 15 years. New mediators of inflammation continue to be identified. The sequencing of the human genome will also provide scientists with many new molecules likely to be involved in the pathogenesis of septic shock; each one may be a target for novel therapies. On the other hand, old cytokines may be 'rediscovered'. For example, the biological role of macrophage migration inhibitory factor (MIF), one of the first lymphocyte cytokines described (in the late 1960s), has been uncovered recently. MIF was found to be a pro-inflammatory macrophage and pituitary mediator that plays a critical role in septic shock.^[51,52] Surprisingly, MIF production is also induced by glucocorticoids and it functions as a counter-regulator of the anti-inflammatory and immunosuppressive effects of steroids on macrophages and T cells.^[53,54] More recent experiments have shown that MIF plays an important role in animal models of glomerulonephritis, arthritis and allograft rejection, indicating that it is a critical mediator of acute inflammation. Anti-MIF strategies may find utility in the management of septic shock or other inflammatory diseases.

Many systems are activated during septic shock. Blocking the coagulation, complement or kinin cascades is another attractive approach for the

mice confirmed the importance of these 2 molecules in the pathophysiology of septic shock.^[48,49] No data are yet available on the effect of anti-LBP or anti-CD14 antibodies in humans with sepsis.

management of patients with sepsis. Studies using antithrombin III or inhibitors of the tissue factor pathway are currently in progress. Inhibition of the selectins or integrins is yet another potential strategy for preventing the activation of inflammatory or immune cells.

Conversely, several studies have shown that the immune cells of patients with sepsis are hyporesponsive or deactivated, suggesting that critically ill patients may be immunosuppressed, especially in the post-acute phase of septic shock.^[55] These observations have led some investigators to promote an alternative strategy using agents such as interferon- γ , granulocyte colony-stimulating factor or granulocyte-macrophage colony-stimulating factor to boost immune function and reverse cellular deactivation in these patients. Preliminary data suggest that treatment with interferon- γ may improve immune function in selected medical and surgical intensive care unit patients, but double-blind placebo-controlled studies are needed to examine further the efficacy and tolerability of such interventions.^[56]

3. Conclusions

Although the results of recent clinical trials of adjunctive therapy for sepsis have been discouraging, several potential treatment strategies are available and could be explored to improve the outcome of such patients. The success of these approaches will rely both on improvement of our scientific knowledge and ability to develop agents for clinical trials, and on improved selection of suitable patients for these trials.

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Review

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Bloodstream Infections: Epidemiology, Pathophysiology and Therapeutic Perspectives

Introduction

Bloodstream infections (BSI) are associated with a high mortality, despite the introduction of new antimicrobial agents and progress in supportive therapy. Epidemiological studies frequently use results of blood cultures to evaluate the incidence and outcome of BSI. However, the presence of bacteria in the bloodstream, defined as bacteremia, is insufficient in itself to assess the severity of the infection. In an attempt to better define the spectrum of disorders associated with sepsis, Bone et al. (1991) have proposed a diagnostic system based on progressive stages of BSI: bacteremia (presence of positive blood cultures); sepsis (clinical evidence suggestive of infection plus signs of a systemic response to the infection); sepsis syndrome (clinical diagnosis of sepsis as outlined above, plus evidence of altered organ perfusion) and septic shock (clinical diagnosis of sepsis syndrome plus hypotension) [1]. This classification allows us to allocate patients to clinical trials based on clinical criteria, and to evaluate the severity of the infection [2]. Using this system we evaluated all patients who had been admitted between 1992 and 1993 to São Paulo Hospital (University Hospital of Escola Paulista de Medicina, UNIFESP - São Paulo), with positive blood culture and observed a mortality rate of 33% in patients with bacteremia, 36% in those with sepsis, 72% in sepsis syndrome and 78% in septic shock [3].

The pathophysiologic events seen in sepsis may also be triggered by non-infectious stimuli, such as trauma, burns and acute pancreatitis. In a Consensus Conference, sponsored by the American College of Chest Physicians and the Society of Critical Care Medicine, the term "systemic inflammatory response syndrome" (SIRS) was proposed to embrace the common inflammatory pathways involved in all these different events [4], while the term sepsis is to be restricted to SIRS caused by infection. Moreover, they suggested that the term sepsis syndrome should no longer be used as it is confusing and ambiguous [4]. Prospective clinical studies are currently being conducted to validate these criteria, and it has already been shown that the consensus criteria for SIRS were very sensitive but lack in specificity [5]. The need for more uniform concepts is of paramount importance for the design and evaluation of new therapeutic strategies.

Epidemiology

The incidence and etiology of BSI have changed over recent years, justifying further epidemiological studies. One of the most interesting studies in the field came from McGowan, Barnes and Finland who, between 1935 and

1972, evaluated BSI at the Boston City Hospital [6]. This study allows us to evaluate the impact of the introduction of antimicrobial agents on the epidemiology and mortality of BSI. A fourfold increase in incidence was observed and the etiology was altered dramatically during this period. In 1935, about 85% of cases were due to gram-positive bacteria, with *Streptococcus* spp. and *Staphylococcus aureus* accounting for 90% of the fatal cases, whilst in 1972 gram-positive and gram-negative bacteria were found in equal numbers. The increasing incidence of gram-negative bacteria has also been observed by McCabe and Jackson [7] and Kreger et al. [8], who reported a tenfold increase in the incidence of gram-negative bacteremia between 1951 and 1974. In the 1980s a shift towards gram-positive infections was observed in hospitalized patients [9, 10]. The BSI rate due to gram-negative bacilli remained stable, but the rate of gram-positive infections increased [11]. This change was mainly due to an increase in the incidence of BSI by coagulase negative *Staphylococcus* (CNS), *enterococci* and *S. aureus* [11-13]. More recently, *Candida* spp. has emerged as a significant pathogen of BSI, being the fourth most common etiologic agent in some institutions [14].

At the University Hospital of Escola Paulista de Medicina we have observed that the proportion of BSI due to gram-positive bacteria increased from 43 to 54% between 1985-86 and 1992-93. The incidence of gram-positive bacteremia, however, was higher only for coagulase negative *Staphylococcus* infections. This change was mainly due to a decrease in the incidence of gram-negative bacteremia in the latter period. In contrast to reports in the literature, the incidence of BSI was higher in the first period (26/71000) than in the second (14/1000) [3].

The incidence of bacteremias is higher in major teaching hospitals and lower in smaller non-teaching hospitals [15]. Although this remains true, a recent survey showed that the incidence of BSI was increasing more rapidly in non-teaching hospitals [11].

Bacteremias can be hospital or community acquired. Hospital-acquired infection is defined as not having been present or in incubation at the time of admission [16]. Hospital-acquired bacteremia represents over fifty percent of all bacteremic episodes and is associated with a higher morbidity and mortality [12, 17-19].

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Mortality related to bacteremia is about 30% [6, 17, 20, 21]. The factors associated with poor outcome are: extremes of age, hospital-acquired infections, site of origin of infection (respiratory tract) and some etiologic agents (such as *Pseudomonas* spp.), the presence of shock, the severity of the underlying disease and inappropriate use of antimicrobial agents [7, 8, 17, 20, 22–24]. Among these factors, the presence of severe underlying disease is of paramount importance. McCabe and Jackson [7] classified the underlying diseases into three groups: rapidly fatal, ultimately fatal, and non-fatal and observed a mortality of 91, 66 and 11%, respectively. These data have been confirmed by several authors [8, 19, 22, 25–27].

Another important variable associated with outcome of sepsis is the appropriateness of antimicrobial therapy [17, 22, 23]. In one series, patients treated with antimicrobial agents to which the bacteria causing BSI showed *in vitro* resistance had a mortality over three times greater than patients treated with antibiotics to which the etiologic agent showed *in vitro* sensitivity [17].

Using multivariate analysis, we have found that underlying disease, age, appropriateness of antimicrobial therapy, shock and hospital-acquired bacteremia were the factors associated with poor prognosis among 559 episodes of BSI at the Hospital São Paulo, observed in 1985–86 and 1992–93 [3].

The increasing incidence of BSI and its high mortality show the importance of developing new therapeutic strategies. This, however, necessitates a better understanding of the underlying mechanisms involved in the pathophysiology of sepsis.

Pathogenesis

The pathogenesis of sepsis involves complex interactions between the invading bacteria and the defense mechanisms of the human or animal host organism. Interactions with bacteria already begin at birth; infection, however, is a relatively infrequent event, because the invading microorganisms can be adequately dealt with by the defense of the host. In cases of impaired immunity, such immune suppression due to underlying diseases or therapy frequent in the hospital environment, facilitates infection. Paradoxically, the host immune and inflammatory responses, essential for the control of infection, also contribute to the deleterious sequelae seen in sepsis.

The ability of bacteria to mount an inflammatory response is associated with biologically active components present in the bacteria. In the case of gram-negative bacteria, endotoxin (lipopolysaccharide [LPS]) is of particular interest. Endotoxin is an integral part of the outer bacterial membrane. It is vital for bacterial survival, conferring stability to the bacterial membrane, selective permeability and resistance to biliar salts and antimicrobial agents [28, 29]. The release of endotoxin from bacteria takes place after death and lysis of the cell. Today, we know that a small amount of LPS can be liberated also during life and growth

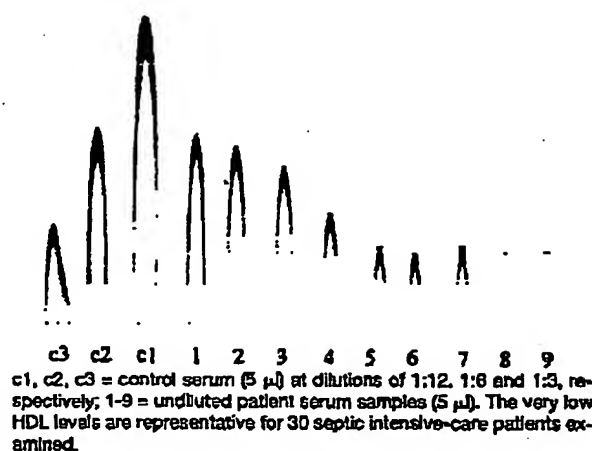


Figure 1: Circulating levels of HDL in a patient with severe infection. Quantitative rocket immunoelectrophoresis analysis of HDL levels in the serum of a healthy person (control) and in serum samples obtained daily from an intensive-care patient with severe gram-negative bacterial infection.

of the bacteria. Administration of purified LPS in experimental animals induces fever, diarrhea, intravascular coagulation, hypotension and shock, mimicking the clinical picture seen in sepsis. Therefore, LPS is thought to be the major toxic component of gram-negative bacteria; hence, it has an important role in the pathophysiology of sepsis [29–31].

Chemically, endotoxins are lipopolysaccharides, consisting of the O polysaccharide, the core oligosaccharide, and a lipid part, designated lipid A [31–33]. The O polysaccharide exhibits a high structural and antigenic diversity among different bacterial strains, allowing bacteria to be classified into O serotypes. The core oligosaccharide shows less variability and the lipid A moiety is antigenically similar in all pathogenic gram-negative bacteria [31]. As a result, antibodies raised against the lipid A of one bacterial strain will react with the lipid A of most pathogenic gram-negative bacteria [34]. This is of great interest as lipid A is the toxic component of LPS. Administration of free or synthetic lipid A into experimental animals reproduces the features of endotoxemia [32, 35].

Intravenously administered LPS circulates until it is cleared by the reticulo-endothelial system. The half-life time of clearance depends on the form (smooth or rough form) and on the state of aggregation of LPS. The clearance of LPS is effected mainly by the liver and to a lesser extent by the spleen. The uptake of LPS in the liver is effected by Kupffer cells. From Kupffer cells LPS is translocated to hepatocytes. Experiments in rats and mice have shown that, in the case of R-form LPS, hepatocytes also participate in the primary uptake from the circulation, and thus bypass Kupffer cells. Hence hepatocytes represent the final site of LPS accumulation in the liver, from where LPS is excreted via the bile into the intestine. In the framework of these experiments it was shown that a part

of the LPS is excreted via the lung, in the form of LPS-carrying macrophages, which migrate into the alveolar and bronchial space. The removal of LPS from the organism is very slow. It takes place during many weeks and is independent of the dose of LPS administered. It is interesting that despite the long persistence of LPS in the organism, only minor alterations in chemical structure were found. These, however, did not biologically affect activity, since LPS isolated from rat liver was highly toxic in different biological tests [36, 37].

The interactions of LPS with cells and subsequent activation may proceed directly and in principle does not require the help of accessory plasma components.

The major part of circulating LPS is bound to HDL. HDL-bound LPS has much lower affinity for cells. This is also true for macrophages, which are primary targets of LPS. It has been postulated that the binding to HDL lowers the biological activity of LPS by retarding its cellular uptake. It may be looked upon as an indirect mechanism of detoxification of LPS [37]. In this respect is interesting that patients suffering from sepsis exhibit very low HDL levels (Figure 1) (Freudenberg, Beger, Galanos, unpublished observation). In a recent clinical trial in human volunteers, administration of recombinant HDL significantly reduced the cytokine release and expression of surface antigens in response to LPS [38]. Hence, substitution with HDL may be of clinical use in the future.

Another protein that interacts with LPS and which is currently of particular interest is the lipopolysaccharide binding protein (LBP) [39]. LBP is an acute phase reactant which facilitates the binding of LPS to soluble(s) CD14 in plasma and to membrane-bound (m) CD14 on target cells of LPS [40–42]. The LBP mediated binding to monocytes/macrophages results, as shown in *in vitro* studies, in an enhanced activation and subsequent formation of pro-inflammatory cytokines [161–163]. Activation of cells that lack CD14 seems to be mediated by the complex of LBP-LPS-CD14 [41]. Excess CD14 suppresses, however, LPS-induced cytokine production by monocytes. Antibodies to LBP [43] and CD14 [40] have been reported to inhibit LPS activity and toxicity. The generation of LBP-deficient (LBP-/-) mice lately allowed the assessment of the importance of LBP in the development of LPS effects *in vivo* [164, 165]. It was shown that in normal, healthy mice LBP-/-, LPS responses may proceed in the complete absence of LBP, albeit to a lower degree than in wild-type mice. However, the characteristic hyper-responses which are induced by LPS in mice that had been made hypersensitive to LPS by prior treatment with bacteria, proceed exclusively in the presence of LBP. Interestingly, while less susceptible to the effects of LPS, LBP-/- mice were shown to be more susceptible towards peritoneal infection with *Salmonella typhimurium* [164]. Thus, during infection with gram-negative bacteria, a dual role for LBP may be envisaged; a protective role since, in the presence of LBP, the infected host would sense small amounts of LPS and react against infection at a time when the number of bacteria is

still small. On the other hand, failure to restrict the infection at an early stage would lead to a multiplication of the bacteria, resulting not only in larger amounts of LPS but also in the development of hypersensitivity. In this case, the toxic activity of LPS would be greatly enhanced by LBP and would have hazardous consequences for the infected host. Nowadays, it is generally accepted that LPS toxicity is mediated by the host response and endogenous mediators are finally responsible for the different pathophysiological activities of LPS. The availability of inbred mice genetically resistant to the lethal effects of LPS have been very useful in elucidating this point. It has been shown that administration of LPS to resistant mice, C3H/HeJ, does not result in lymphocyte and macrophage activation, and the animals survive otherwise lethal amounts of LPS without signs of toxicity [44]. The transfer of reticuloendothelial cells from sensitive mice (C3H/HeN) to resistant mice (C3H/HeJ) renders the latter sensitive to LPS [45]. Freudenberg, Galanos and coworkers have shown that macrophages are the cells mediating LPS toxicity [46, 47].

LPS-activated macrophages secrete a series of mediators, of which TNF- α plays a pivotal role in endotoxic shock. Beutler, Cerami and coworkers were the first to recognize TNF as a primary mediator of LPS by showing that anti-TNF- α antibodies partially protected mice from the lethal effects of LPS [48, 49]. Complete protection from the lethal activity of LPS by anti-TNF antibodies was also demonstrated in D-galactosamine-sensitized mice, a model in which TNF is the sole mediator of lethality [47]. With the advent of recombinant TNF- α it has been shown that TNF- α administration mimics many pathological alterations seen in sepsis [50, 51]. These experimental data have been supported by clinical investigations reporting higher levels of circulating TNF- α in patients with sepsis who had died compared to those who had survived [52–55].

TNF- α is mainly produced by macrophages stimulated by LPS or other agents. TNF- α is not produced constitutively. It can be detected in the circulation after LPS injection, reaching peak values after 60 to 90 min, and disappearing over the following hours. Similar kinetics have been described in man, rabbits, rats and mice [56–59]. Synthesis is regulated at the transcriptional and post-transcriptional levels [60, 61]. TNF is subject to complex mechanisms of up and down regulation by natural-killer (NK) and T-cell-derived cytokines. In this context, IFN- γ and TNF- α act synergistically by activating macrophages, while IL-4 and IL-10 inhibit macrophage activation and the subsequent production of cytokines [62–68]. Interestingly, monocytes are also a source of IL-10. It has been shown that production of IL-10 by monocytes downregulates their production of pro-inflammatory cytokines, thus IL-10 exerts an autoregulatory role on macrophages [65].

Other cytokines induced by LPS are IL-1 and IL-6, which together with TNF- α are referred to as pro-inflammatory cytokines. There is considerable overlapping of biological activities between TNF and IL-1 [69]. Also, administration of rIL-1 induces shock in rabbits [70]. It was shown that

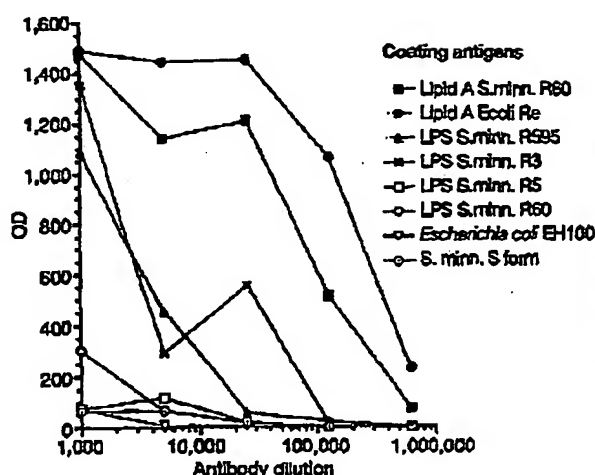


Figure 2: Binding activity of monoclonal anti-lipid A antibodies (clone 31, IgG2b) to lipid A and LPS preparations with different stages of completion of the core. Similar result was obtained with monoclonal IgM antibodies.

TNF and IL-1 [70] and TNF, IL-1 and LPS [71] interact to cause hypotension in these animals.

The role of TNF- α (and IL-1) as a mediator of endotoxic shock can be illustrated by its effect on endothelial cells. TNF- α and IL-1-activated endothelial cells secrete other mediators such as IL-1, PAF, prostaglandins, thromboxane and NO, as well as producing pro-coagulant factors [69, 72-74]. The LPS-induced activation of the coagulation cascade proceeds through intrinsic (Hageman factor XII) and extrinsic pathways [75]. Activated factor XII causes the conversion of pre-kalikein to kalikrein, which in turn cleaves high molecular-weight kininogen releasing bradykinin. The consequent small vessel obliteration, vasodilatation, increased permeability with leakage of fluid and inflammatory cells, together with intravascular disseminated coagulation, are the main alterations that underlie the complex clinical picture of sepsis. In the lungs, these alterations result in the Acute Respiratory Distress Syndrome (ARDS). In rats, the presence of endotoxin-carrying mononuclear cells in small pulmonary vessels of animals injected 3 days earlier with LPS could be detected [76].

The lethal effect of endotoxin in experimental animals can be enhanced under a variety of conditions, such as infections [77] or the presence of growing tumors [78]. Under these conditions hypersensitive animals produce enhanced amounts of cytokines (TNF- α) upon LPS challenge. The hypersensitivity to LPS induced by infection is mediated by IFN- γ [79-81]. Thus IFN- γ produced in the course of infection is potentially a risk factor in the development of LPS shock. Sensitization to endotoxin may also be induced by a number of hepatotoxic agents, such as D-galactosamine (D-GalN) [82]. In all situations mentioned above, sensitization to LPS was paralleled by a sensitization to the lethal activity of TNF- α [51, 77, 78].

Table 1: Toxic properties of killed gram-negative and gram-positive bacteria for D-GalN sensitized endotoxin-resistant C57BL/10 ScCr mice.

Preparation	Approx. lethal dose 50 (μ g)
LPS:	
<i>S. abortus equi</i>	> 1000
Bacteria	
<i>S. abortus equi</i>	20
<i>Salmonella typhimurium</i>	20
<i>Staphylococcus aureus</i>	10
<i>Propionibacterium acnes</i>	100
<i>Mycobacterium phlei</i>	50

Groups of mice received D-galactosamine (20 mg) and different amounts of each preparation injected i. v. as a mixture. The absence of lethality in the endotoxin-resistant mice receiving an excess of LPS (1000 μ g) and the high lethal toxicity of the bacteria for these mice is evidence for the presence of toxic, non-LPS components in these bacteria.

On the other hand, minute amounts of LPS render the animals hyporesponsive to a subsequent LPS challenge, a phenomenon known as tolerance, which can be divided into early and late phase tolerance [reviewed in 83]. The two types of LPS tolerance seem to be based on fundamentally distinct mechanisms. Early phase tolerance appears within hours (or a few days) after a single injection of LPS and disappears over the subsequent days. Late phase tolerance occurs after several days and persists for weeks or even months. In contrast to early phase, which can be produced with serologically unrelated LPS, late phase tolerance is related to the production of O-antibodies, and is thus specific for a particular LPS serotype.

Early phase tolerance can be induced by S- and R-form LPS and also by free lipid A [84]. Macrophages play a central role in the development of endotoxin tolerance [47]. As shown earlier, macrophages from tolerant animals are found to be refractory to the stimulatory activity of LPS. Consequently they exhibit a suppressed production of many mediators, including endogenous pyrogen (IL-1) [85], prostaglandins [86], CSA [87] and TNF- α [88]. Madonna and Vogel [89] showed a marked increase in bone marrow-derived macrophage progenitor cells in association with LPS tolerance. Freudenberg and Galanos [90], using the D-galactosamine model, demonstrated that macrophages are involved in the induction of LPS tolerance. We have observed that in D-GalN-sensitized mice, tolerance to the lethal effects of LPS developed in parallel with the suppression of LPS-induced TNF. In normal mice, a state of hypersensitivity to LPS (20 h after pretreatment) precedes the onset of tolerance (present 72 h after pretreatment). This is an interesting phenomenon since hypersensitivity is present at a time when TNF induction is suppressed (Salomão, Freudenberg and Galanos, manuscript in preparation). D-GalN-sensitized mice made tolerant to LPS were also found to be tolerant to TNF- α , which indicates that tolerance, in this model, is not only due to an unresponsiveness of the macrophages to LPS,

but also to an unresponsiveness of the mice to the activity of the mediator(s) [47, 90]. However, in normal mice and in other models of sensitization, cross-tolerance between LPS and TNF- α is not usually found. Vogel et al. [91] have shown that the combination of TNF- α and IL-1 can substitute LPS in the induction of tolerance. So far the data suggest that the qualitative and quantitative aspects of tolerance may vary considerably, depending on the model of lethality employed [79].

TNF- α is also induced by gram-positive bacteria, and by non-LPS components of gram-negative bacteria. Table 1 shows that both types of bacteria induce lethality in D-GalN sensitized, LPS-resistant mice and as shown earlier, this lethality can be inhibited completely by anti-TNF- α antibodies [92]. The involvement of TNF- α in the pathogenesis of gram-negative and gram-positive experimental infections [79, 92] and its presence in patients with BSI caused by these bacteria [53, 93] suggest common pathophysiological pathways for both infections. On the other hand, an increasing body of evidence suggests an essential role for TNF- α in the host defense against infections, such as leishmaniasis [94], BCG [95], histoplasmosis [96], listeriosis [97] and even in sepsis itself [98, 99].

Therapeutic Perspectives

The therapeutic perspectives may be roughly divided into: development of new antimicrobial agents, immunological approaches and better support therapy.

The appropriateness of antimicrobial use is of paramount importance in the clinical management of sepsis. Antimicrobial agents to which the infecting bacteria are sensitive *in vitro* significantly reduce mortality compared to agents to which the bacteria are resistant [17, 22, 23]. At the University Hospital of Escola Paulista de Medicina, we observed a mortality of 21 and 57.1%, respectively [19]. Substitution of antimicrobial agents, to which the bacteria were resistant *in vitro*, for one to which bacteria were sensitive, reduced the mortality rate in such patients [19]. The emergence of multi-resistant gram-negative and gram-positive bacteria [100–102] has complicated antimicrobial therapy especially in intensive care settings. Development of new drugs to treat such resistant bacteria is therefore imperative. However, such drugs must be used judiciously, as further indiscriminate use will only be followed by the emergence of new resistant strains.

Immunological approaches are aimed both, at interfering with the ongoing pathophysiological response to infection or at preventing its development. Results of preliminary investigations are encouraging, although the clinical results are still conflicting. So far they have been aimed at neutralizing LPS/lipid A, blocking its binding to macrophages and inhibiting the release or action of cytokines, such as TNF- α and IL-1 and cytokine-induced mediators thereof, such as NO.

The lipid A/inner core region of the LPS molecule is antigenically preserved among many pathogenic gram-negative

bacteria and is the part of LPS responsible for endotoxic activity. The prospects of preparing antibodies directed against common structures, that were capable of neutralizing the toxic effects of LPS of serologically unrelated gram-negative bacteria, seemed very exciting, and attracted the interest of many workers in the field. Antibodies to lipid A, obtained for the first time in 1971 [103], were found to exhibit a wide cross-reactivity with the free lipid A of unrelated bacteria [31, 34]. Also, a large number of studies using anti-sera obtained after immunization with rough mutants of *Escherichia coli* and *Salmonella minnesota* (mainly Re and Rc LPS chemotypes) have shown cross-binding to and protection against the toxic effects of LPS, as well as protection against infection induced with different bacteria [104–106]. One of the questions that remained controversial until now is whether these antibodies were able to recognize and bind to the lipid A/inner core as part of the intact LPS molecule, thus protecting experimental animals against LPS toxicity and bacterial infections, or if the protection was related to other mechanisms triggered by immunization. Using immunoblotting, we observed that the cross-reactivity of polyclonal antibodies was directed towards the less complex R-forms of LPS, i.e. the rough compounds. In a recent review, Greisman and Johnston considered the possibility that protection in the above-mentioned studies may have been due to mechanisms such as natural and polyclonally stimulated antibodies to O-specific antigens, presence of acute phase proteins in the antiserum and contamination of the antiserum with endotoxin [107].

The protective effect of anti-lipid A/core polyclonal antibodies observed in experimental infections by some authors [108–111] led to their use in clinical trials. The results obtained in one prospective, randomized, double-blind study, using polyclonal anti-core antibodies obtained by immunizing with the LPS of *E. coli* J5 were encouraging. A 37% reduction in mortality in patients with gram-negative bacteremia and a 39% reduction in those with shock was seen in the treated group compared to placebo [112]. However, the specificity of such antibodies for LPS and their protective effect during infection could not be confirmed in other studies [113–115]. Likewise a prospective, randomized, double-blind study showed no reduction in mortality in patients administered anti-*E. coli* J5 antibodies [116]. In another clinical trial comparing the prophylactic effects of intravenous standard immunoglobulin and core-lipopolysaccharide immunoglobulin in patients at high risk of post-surgical infection, no differences in gram-negative infections or their complications could be observed [117]. With the advent of monoclonal antibodies, less conflicting results were expected. Thus, monoclonal antibodies to lipid A have been found to have a wide range of cross-reactivity to LPS [118–123]. Other monoclonal antibodies to lipid A, however, have been found to be extremely restrictive in their binding activity [124–126]. In our hands, the binding of anti-lipid A monoclonal antibodies to LPS correlated inversely with the stage of completion of the core (Figure 2).

Promising clinical results have been reported with monoclonal antibodies to lipid A, HA-1A and E5, in two prospective, randomized, double-blind studies [127, 128]. In both studies the antibodies conferred a protective effect in one subgroup of patients, but not in the entire study population. In one study, a greater protective effect was seen in patients with severe disease, i.e. with shock, while in the other only patients without shock benefited from the treatment. Further investigations with HA-1A and E5, did not confirm these benefits [129, 130], and raised questions regarding the binding and protective properties of these antibodies. Warren et al. [131] did not succeed in neutralizing LPS-induced mitogenic effect or cytokines with antibody preparations HA-1A or E-5. In another report [132], neither protective effects to LPS toxicity nor suppression of TNF- α induction was shown. All these conflicting results have raised concern about the usefulness of further pursuing such studies. Recently, Di Padova and coworkers described monoclonal antibodies to core epitopes with extended cross-reactivity to LPS. Interestingly, this Mab was able to neutralize LPS-induced cytokines *in vitro* and to suppress endotoxic activity, assessed by fever measurement in rabbits and mortality in D-GalN-sensitized mice [133]. Neutralization of TNF- α and IL-1 protects animals from the toxic effects of LPS [47, 49] and otherwise fatal bacteraemias. Phase I clinical trials with murine anti-human TNF- α antibodies have revealed a half-life time of 52 h and few collateral effects [134]. However, a prospective, double-blind, randomized clinical trial with monoclonal anti-human TNF failed to reduce mortality in patients with sepsis [135]. This failure may be due to: 1) the complexity of pathogenesis, which involves many mediators; 2) administration of antibodies when the cascade of inflammation has already been triggered; or 3) neutralization of possible protective effects of these cytokines in the overall outcome of infection. Although in sepsis the presence of TNF- α has been associated with deleterious effects and a poor prognosis, and its inhibition in experimental studies has led to protective effects in animals, there is an increasing body of evidence that the presence of TNF- α is crucial for host defense against infection. This role has been shown in infections due to *Listeria monocytogenes* [97], BCG [95], cutaneous leishmaniasis [94] and also in experimental models of sepsis, such as *S. typhimurium* (Mitov, Freudenberg and Galanos, unpublished observations). Thus anti-TNF- α antibodies either did not protect or, on the contrary, enhanced the lethality of experimental peritonitis [136, 137]. Also, decreased production of TNF- α by peritoneal macrophages of patients undergoing continuous ambulatory peritoneal dialysis has been associated with a higher incidence of peritonitis [138]. Blood cells of heavily infected patients exhibit reduced TNF-response to LPS [139]. Moreover, some authors have failed to find a link between circulating levels of cytokines and outcome, while others have found the presence of circulating TNF- α to be associated with an improved host response and better outcome in sepsis [98, 99]. Recently, the

use of a TNF- α inhibitor, a dimer of an extracellular portion of the TNF receptor and the Fc portion of IgG1, enhanced the mortality of patients with sepsis [140]. Mortality was 30% in the placebo group and as high as 53% in patients with the highest dose of sTNF-R/IgG [140], further supporting a role for TNF- α in host defense against bacterial infection. It has also been shown that deletion of the TNF gene or of TNF-R protected experimental animals from the toxic effects of LPS, but impaired the clearance of bacteria from the lungs [141] and increased lethality of infection [142].

The use of corticosteroids in sepsis remains controversial. In prospective, double blind clinical trials treatment with corticoids did not improve outcome in patients with sepsis. On the contrary, there were more infectious complications in patients treated with corticosteroids than in the control group [143, 144]. Experimentally, corticosteroids have a protective effect in endotoxic shock. A possible explanation for these divergent findings is the timing of corticosteroid administration in experimental models compared to the clinical setting. Experimentally, protective effects are seen when corticosteroids are injected before or simultaneously with LPS. In septic patients, corticosteroid administration may be delayed and only given when the inflammatory cascade response has already been triggered. In support of this hypothesis, Galanos et al. [145] found that dexamethasone affords complete protection against the toxic effects of LPS in D-GalN sensitized mice, when administered before LPS. However, there is no protection when it is injected 15 min or longer after LPS. Pretreatment with dexamethasone affords protection against LPS by inhibiting TNF- α production, but has no protective effect against activity of TNF- α , once this has been formed. It is concluded that corticosteroid-mediated protection is due to the inhibition of the release of mediator(s), an effect that is lost once the production of mediator(s) is under way [145].

CD14, believed to represent an LPS receptor, together with LBP enhances the sensitivity of the cells to LPS by the order of 100 to 1000 [39, 40] and is, therefore, a potentially important target of therapeutic strategies. Blocking of the binding of the LPS-LBP complex to CD14 by monoclonal antibodies has been shown to decrease the binding of LPS to macrophages, neutrophils and even endothelial cells [40, 41]. Antibodies to LBP were reported to have a protective effect against the toxic effects of LPS and lipid A in experimental animals. This effect is believed to be due to a significant decrease in TNF- α production [43]. Some proteins released by neutrophils have been reported as binding to and inactivating the LPS. The most important of these is the bactericidal/permeability-increasing protein (BPI), a 55/60 kD protein, which shares a 45% homology with LBP [146]. Besides neutralizing LPS, BPI is cytotoxic against gram-negative bacteria. Activity is due to the 25 kD N-terminal region, which has been produced as a recombinant 23 kD fragment (rBPI23) [147]. Injection of rBPI23 protected mice from LPS-induced tissue damage and death

(93% survival in rBPI23 group vs 13% in control group), and reduced LPS-induced TNF and IL-1 production [148]. Also, a number of lipid A analogues with a low toxicity may qualify as potentially competitive antagonists of LPS. One of these, lipid X, suppresses LPS activation of neutrophils and protects against LPS toxicity. The lipid A of *Rhodopseudomonas sphaeroides*, a lipid A of very low toxicity [149], also protected experimental animals against the fatal effects of LPS [150]. In addition, this molecule was able to induce tolerance to LPS [151].

NO, a potent vasodilator, plays a pivotal role in septic shock. It is produced by NO synthase, which is found in constitutive (type I and type III) and inducible (type II) isoforms. Constitutive isoforms (cNOS) are expressed in neuronal (type I) or endothelial (type III) cells and inducible (iNOS) are expressed in many cells, including macrophages [152]. While cNOS are responsible for the reactions of organic homeostasis, iNOS is involved in situations of stress, such as those induced by LPS or cytokines [153]. Non-selective inhibition of the inducible isoform appears to explain the lack of beneficial effects seen in experimental studies using NO synthase inhibitors [154]. One inhibitor, N-monomethyl-L-arginin (L-NMMA), has been used in clinical trials. In 12 patients with sepsis, L-NMMA induced a significant increase in mean arterial pressure and systemic and pulmonary resistance, but also caused a reduction in cardiac output and tissue oxygen uptake [155]. Methylene blue, an inhibitor of guanilato cyclase, ameliorated hemodynamic parameters in two patients without preventing death [156]. Selective inhibitors of the iNOS could present promising therapeutic options. S-methylisothiourea (SMT) and aminoguanidine, two inhibitors of NOS with selectivity of iNOS, prevented rats from pulmonary transvascular flux after injection of LPS [157]. Prolonged survival of rats infected with *E. coli* was observed with SMT, while a high dose of NG-nitro-L-arginine methyl ester (L-NAME), an inhibitor of constitutive and inducible forms of NOS, significantly shortened survival times [158]. Enhanced lethality with L-NAME was also observed in mice injected intraperitoneally with *E. coli* [159]. However, even S-ethylisothiourea and 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine, two iNOS inhibitors, were shown to increase lethality of rats exposed to LPS in one study [160]. Pretreatment with L-arginine could prevent mortality without affecting the iNOS-dependent NO production, thus suggesting that toxicity was due to inhibition of other NOS isoforms (neuronal or endothelial). It has been suggested that iNOS inhibitors of greater selectivity may be needed for therapeutic use [160].

Other approaches for the treatment of sepsis are currently under investigation. Overall results are still discouraging. This may be due to the complexity of the pathogenic process of sepsis. However, a better understanding of the mechanisms involved in this dramatic medical emergency open up new perspectives for therapeutic approaches, in addition to antimicrobial treatment, that may result in a reduction in the high mortality associated with this syndrome.

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Clinical Trials of a Matrix Metalloproteinase Inhibitor in Human Periodontal Disease

ROBERT A. ASHLEY^a AND THE SDD CLINICAL RESEARCH TEAM

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ABSTRACT: After demonstration by Golub *et al.* of the ability of the tetracyclines to inhibit elevated collagenolytic activity in animal models of periodontal diseases, a clinical development program was initiated to demonstrate the potential of a subantimicrobial dose of doxycycline (SDD) to augment and maintain the improvements in clinical parameters of adult periodontitis (AP) afforded by conventional nonsurgical periodontal therapy. Clinical trials were carried out in which a number of different SDD dosing regimens and placebo were compared in patients administered a variety of adjunctive nonsurgical therapies. Measured parameters included levels of collagenase activity in gingival crevicular fluid (GCF) and gingival specimens, clinical attachment levels (cALv), probing pocket depths (PD), bleeding on probing (BOP), and subtraction radiographic measurements of alveolar bone height. When used as an adjunct to either scaling and root planing or supragingival scaling and dental prophylaxis, SDD was shown to reduce collagenase levels in both GCF and gingival biopsies, to augment and maintain cALv gains and PD reductions, to reduce BOP, and to prevent loss of alveolar bone height. These clinical responses arose in the absence of any significant effects on the subgingival microflora and without evidence of an increase in the incidence or severity of adverse reactions relative to the control groups. It is proposed that one of the mechanisms of action of SDD is as an inhibitor of pathologically elevated MMPs, including neutrophil and bone cell collagenases (MMP-8 and MMP-13), which are associated with the host response in chronic AP, and that SDD provides a novel systemic approach to the management of AP.

INTRODUCTION

Periodontitis is the most common cause of adult tooth loss in the United States.¹ A recurring and site-specific condition, periodontitis involves inflammation of the gingiva together with loss of clinical attachment caused by the destruction of the periodontal support structures and alveolar bone.^{2,3}

Although bacteria are necessary for initiating periodontitis, host responses are in large part responsible for the destruction of the periodontal support structures.³ In patients with periodontitis, pathologic overactivity of host-derived matrix metalloproteinases (MMPs) occurs in response to the bacterial infection in the periodontal tissues. This leads to the excessive destruction of collagen, the primary structural component of the periodontal matrix.⁴ In turn, MMP-mediated destruction of connective tissue collagen leads to gingival recession, pocket formation, and tooth mo-

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bility. In the absence of appropriate therapy, tooth loss may occur in advanced disease.

Commonly used therapies for treating periodontitis include mechanical procedures that control the localized bacterial infection by physically removing plaque and calculus. Although mechanical procedures are frequently effective in slowing the progression of periodontal disease, clinical outcomes may be suboptimal in some patients because these interventions may have only a limited effect on pathologic host responses. New strategies for managing destructive periodontal diseases aim to reduce the bacterial load while simultaneously suppressing the host responses that lead to tissue destruction. This "two-pronged" approach to treatment utilizes mechanical procedures as first-line strategies, with host-modulating pharmacotherapies as adjuncts to first-line treatments.

Recently, attention has focused on the tetracycline antibiotics and their unique ability to inhibit the activities of tissue-destructive MMPs. In a series of landmark studies, Golub and colleagues demonstrated that tetracyclines, such as doxycycline, inhibit collagenolytic activity in gingival tissues.^{5,6} Significantly, this anticollagenolytic action of doxycycline occurs at doses below those required for antimicrobial effectiveness.⁷⁻⁹ Doxycycline has been shown to inhibit MMP-8, the predominant MMP responsible for periodontal destruction, in extracts of inflamed human gingival tissue.¹⁰ Treatment with doxycycline, 20 mg twice daily, reduced the excessive activity of MMPs and reduced the degradation of collagen in gingival crevicular fluid (GCF) taken from adult patients with periodontitis.¹¹ In a preliminary clinical study, doxycycline, 20 mg twice daily, inhibited collagenase activity and improved clinical attachment levels (cAI.v) and probing pocket depths (PD) when administered to periodontal patients periodically over a 6-month period compared with placebo.¹² Taken together, these studies suggest that subantimicrobial dose doxycycline (SDD) may have clinical utility as an adjunct to mechanical interventions in the treatment of periodontitis.

We describe here (1) a 12-week, dose-ranging, Phase II study evaluating the effect of SDD on GCF collagenase activity and selected clinical parameters and (2) long-term, Phase III clinical trials evaluating the efficacy and safety of SDD as a systemically administered adjunct to mechanical procedures in the treatment of adult periodontitis (AP). The effect of SDD on the dynamics of the periodontal microflora is also briefly summarized.

METHODS

Dose-Ranging Study

A Phase II clinical trial was conducted by Golub *et al.*¹⁶ to select the optimal dosage of SDD that reduces GCF collagenase activity and improves attachment levels without inducing doxycycline resistance in microorganisms, and part of the study is summarized as follows. An institutional review board approved the study protocol; patients included in the study provided informed written consent. A total of 75 adult patients with active periodontitis (i.e., exhibiting pockets with repeatedly elevated GCF collagenase activity) were enrolled in a randomized, placebo-controlled, parallel-group, 12-week study. Patients were stratified according to levels of periodontal

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attachment and levels of GCF collagenase activity. At the baseline visit, patients received a dental scaling and prophylaxis and then were randomly assigned to receive various dosing regimens of SDD or placebo for 12 weeks.

Efficacy measures included the reduction in GCF collagenase activity (assessed at baseline and at weeks 2, 4, 8, and 12 of the treatment period) and the change in relative attachment levels (rALv, measured using a Florida Probe with disc attachment). rALv was assessed at baseline and at week 12 of the treatment period. Subgingival microbial samples were harvested at week 12; and sensitivity to doxycycline was determined by standard disk diffusion methodology using a doxycycline-impregnated (30- μ g) disk.

Mean values for GCF collagenase activity were calculated using sample site as the unit of analysis. Mean values for rALv were calculated and used to compare changes occurring over the entire study for each treatment group. Changes from baseline and intergroup differences were tested for statistical significance using analysis of variance or covariance as appropriate.

Efficacy and Safety Studies

To evaluate the safety and efficacy of adjunctive SDD in the clinical setting, four randomized, placebo-controlled, double-blind, parallel-group, Phase III clinical trials were conducted at multiple dental centers in the United States. An institutional review board at each dental center approved the protocols; patients included in the studies provided informed written consent. In all studies, patients received an oral pathology examination at the screening visit, at the baseline visit, and throughout the designated treatment period. Manual probing measurements of cALv and PD were conducted using a UNC-15 probe at screening, baseline, and throughout the treatment period. A single examiner with extensive experience in conducting clinical trials performed all manual probing measurements on a given patient. Duplicate measurements were compared statistically to ensure that individual examiners at each study site obtained acceptable percent reliability.

In three of the studies, the efficacy of SDD was evaluated in conjunction with supragingival scaling and dental prophylaxis (SSDP). A total of 437 patients with clinical evidence of periodontitis (i.e., at least two tooth sites with cALv and PD between 5 and 9 mm inclusive that bled on probing) were enrolled in the studies. Patients received SSDP (30-minute scaling to remove supragingival plaque and calculus followed by tooth polishing with prophylaxis paste and a rotary instrument) at the baseline visit and thereafter at 6-month intervals. Patients were randomly allocated to receive placebo ($n = 119$), SDD 10 mg once daily ($n = 80$), SDD 20 mg once daily ($n = 119$), or SDD 20 mg twice daily ($n = 119$) for 12 months. Patients were instructed to take study medication once in the morning and once in the evening, 1 hour before eating, at approximately 12-hour intervals. For patients receiving SDD once daily, morning medication contained SDD and evening medication contained placebo.

In a fourth study, the efficacy of SDD was evaluated in conjunction with scaling and root planing (SRP). A total of 190 adult patients with evidence of periodontitis (i.e., cALv and probing PD between 5 and 9 mm, inclusive, with bleeding on probing) in at least two tooth sites within each of two quadrants were enrolled in the study. Patients received SRP at the baseline visit. SRP was performed on the quali-

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fying quadrants by the same therapist at each study center, with up to 1 hour allowed per quadrant, until the tooth and root surfaces were visually and/or tactilely free of all deposits. Patients were then randomly allocated to receive either placebo ($n = 94$) or SDD 20 mg twice daily ($n = 96$) for 9 months.

Patients were evaluated after 6 and 12 months of treatment (SSDP studies) or after 3, 6, and 9 months of treatment (SRP study). In all studies, efficacy measures included (1) the change in cALv from baseline, (2) the change in PD from baseline, and (3) the percentage of tooth sites with bleeding on probing (BOP). Efficacy endpoints were evaluated by manual probing at six sites around each tooth in the full mouth (SSDP studies) or within qualifying quadrants (SRP study). Tooth sites were stratified according to AAP criteria of disease severity at baseline (no disease, baseline PD 0 to 3 mm; mild-to-moderate disease, baseline PD 4 to 6 mm; severe disease, baseline PD ≥ 7 mm). Baseline disease severity was determined as the average of duplicate measurements on a given tooth site by manual probing. If the difference between measurements was greater than 2 mm, a repeat measurement was made, and the two closest measurements were averaged to obtain the baseline value.

In the SSDP studies, tooth sites with rapidly progressing disease (e.g., attachment loss of 3 mm or more from baseline to month 6 as measured by manual probing) were discontinued from the study and subjected to mechanical therapy (typically SRP). Patients continued to receive adjunctive SDD or adjunctive placebo after mechanical therapy for the remainder of the treatment period.

Adverse events were recorded in patient diaries, reported at patient interviews, and reported in monthly phone calls. Laboratory tests were performed at the screening visit and throughout the treatment period.

For microbial assessments, subgingival plaque samples were collected at multiple time points using endodontic paper-points. Typical microscopic (e.g., darkfield microscopy) and culture-based techniques (e.g., enumeration of microbes on selective and nonselective media) were used to determine the proportion of distinct cellular morphotypes and to evaluate shifts in the normal periodontal flora, respectively. Agar dilution or agar gradient elution methods were used to assess the susceptibility of the periodontal microflora to doxycycline and other antibiotics.

All treatment group comparisons were performed on the intent-to-treat population (i.e., the population receiving study medication for at least 1 day and having at least one efficacy measurement). Treatment comparisons were performed using a last-observation-carried-forward algorithm (LOCF) to impute missing data at each time point. In the SSDP studies, patients were evaluated as a single population, with the results presented by treatment group. Treatment group comparisons of the per-site efficacy variables were carried out using generalized estimating equation regression techniques.^{13,14} With respect to tooth sites with rapidly progressing disease, the data collected from sites after local therapy were evaluated separately. For the primary analysis, these data were replaced by imputed values based on LOCF techniques.

In the SRP study, treatment group comparisons were performed on per-patient variables. The least square means (or means expressed as percentages) and standard errors were calculated using an appropriate analysis-of-variance model. Microbial parameters were assessed using two-sample, unpaired *t*-tests.

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For all studies, differences between treatment groups were considered statistical-
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RESULTS

Dose-Ranging Study

Over time, all treatment groups demonstrated reductions in GCF collagenase, with mean values approaching levels associated with healthy gingiva after 4 weeks of treatment.^{15,16} At week 12, mean levels of GCF collagenase were significantly reduced from elevated baseline levels in patients receiving SDD 20 mg twice daily (Group 1; $p < 0.05$). For the group receiving SDD 20 mg twice daily (Group 1), the percent decrease in activity from baseline was 47.3% versus 29.1% for the group receiving placebo (Fig. 1).

The effect of SDD on rALv also is shown in FIGURE 1. Improvements in rALv were greatest in the treatment groups receiving doxycycline 20 mg once or twice daily, throughout the entire 12-week course of the study.

Treatment with SDD for 12 weeks did not lead to the emergence of doxycycline resistance of the subgingival microflora; post-treatment isolates remained susceptible to 3 $\mu\text{g}/\text{ml}$ of doxycycline (a concentration approximating the levels of serum doxycycline following an antibiotic dosage [200 mg/day]).

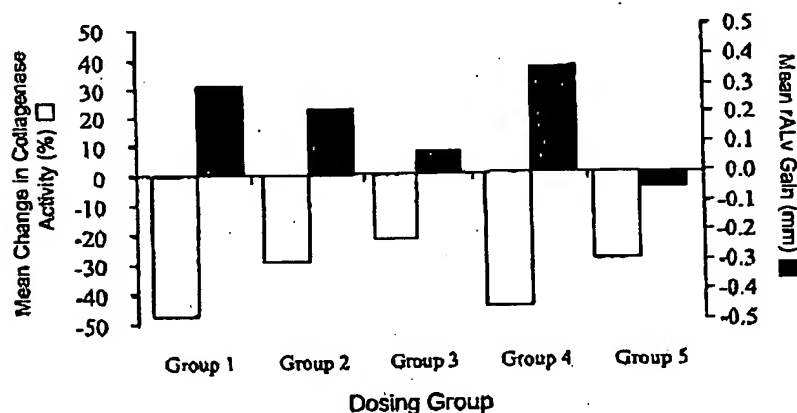


FIGURE 1. Effect of SDD on GCF collagenase activity and rALv. Patients received placebo or SDD as described in the Methods section. Group 1: SDD 20 mg twice daily \times 12 weeks; Group 2: SDD 20 mg twice daily \times 4 weeks then 20 mg once daily \times 8 weeks; Group 3: SDD 20 mg twice daily \times 4 weeks then placebo \times 8 weeks; Group 4: SDD 20 mg once daily \times 12 weeks; Group 5: placebo. SDD = subantimicrobial dose doxycycline; GCF = gingival crevicular fluid; rALv = relative attachment level.

*Efficacy and Safety Studies**Efficacy Results of SSDP Studies*

In all studies combined, no significant differences were demonstrated between the groups receiving either SDD 10 mg once daily or SDD 20 mg once daily and the group receiving placebo. However, significant treatment differences were demonstrated between the SDD 20 mg twice-daily group and the placebo group; these data are presented below.

Across all disease strata, improvements in cALv from baseline were demonstrated for both treatment groups, presumably owing to the course of SSDP administered at baseline and month 6. However, in tooth sites with mild-to-moderate disease (baseline PD 4 to 6 mm), attachment gains demonstrated with adjunctive SDD were significantly greater than attachment gains demonstrated with adjunctive placebo. After 12 months of treatment, the mean attachment gains were 0.67 mm with adjunctive SDD and 0.44 mm with adjunctive placebo ($p < 0.01$ versus placebo). In tooth sites with severe disease (baseline PD ≥ 7 mm), improvements in cALv also were significantly greater with adjunctive SDD than with adjunctive placebo; the mean attachment gains after 12 months of treatment were 1.27 mm with adjunctive SDD and 0.95 mm with adjunctive placebo ($p < 0.05$ versus placebo).

Improvements in PD from baseline were similar to improvements in cALv from baseline. In tooth sites with mild-to-moderate disease, mean reductions in PD were 0.71 mm following 12 months of treatment with adjunctive SDD 20 mg twice daily and 0.46 mm following 12 months of treatment with adjunctive placebo. The difference between the two groups was statistically significant ($p < 0.01$ versus placebo). In tooth sites with severe disease, treatment with adjunctive SDD 20 mg twice daily

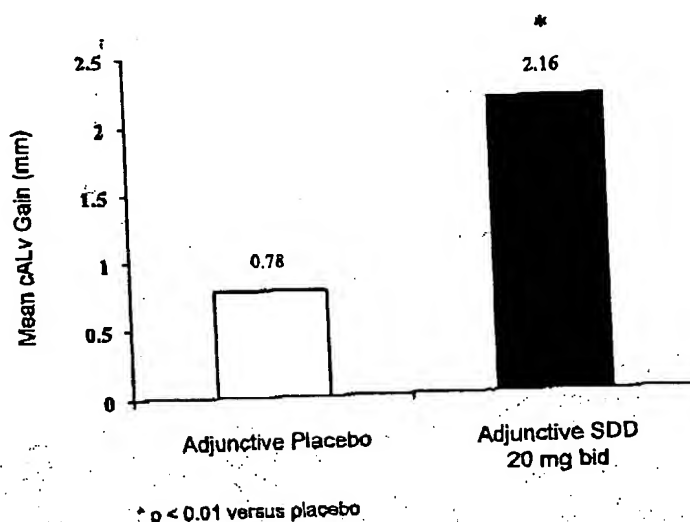


FIGURE 2. Effect of adjunctive SDD on cALv in tooth sites with rapidly progressing AP. Patients received placebo or SDD as described in the Methods section. SDD = subantimicrobial dose doxycycline; cALv = clinical attachment level.

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reduced PD by 1.39 mm, whereas treatment with adjunctive placebo reduced PD by 0.96 mm ($p < 0.01$ versus placebo).

Attachment loss during the first 6 months of the study of 3 mm or more for which an investigator recommended SRP at the 6-month time point was demonstrated in 52 tooth sites in 9 patients receiving adjunctive placebo versus 14 tooth sites in 9 patients receiving adjunctive SDD. This corresponded to a 73% reduction in the incidence of rapid progression of periodontitis. In tooth sites with rapidly progressing disease that were subjected to SRP, the average attachment gain was significantly greater in the adjunctive SDD group than in the adjunctive placebo group (Fig. 2). At 12 months, the mean gain in cALv was 2.16 mm with adjunctive SDD and 0.78 mm with adjunctive placebo ($p < 0.01$ versus placebo).

With respect to BOP, significant improvements from baseline were demonstrated for the adjunctive SDD group after 12 months of treatment compared with the adjunctive placebo group in tooth sites with no disease (SDD [26.3%] versus placebo [31.4%]), in tooth sites with mild-to-moderate disease (SDD [52.3%] versus placebo [60.9%]), and in tooth sites with severe disease (SDD [69.1%] versus placebo, [79.5%]) (all $p < 0.05$).

TABLE 1. Effect of adjunctive SDD on the mean per-patient average change in cALv from baseline (SRP study)^{a,b}

Baseline PD	Mean change (\pm SEM) in cALv from baseline (mm)					
	Month 3		Month 6		Month 9	
	Placebo	SDD	Placebo	SDD	Placebo	SDD
0 to 3 mm	-0.14 (0.03) $n = 92$	-0.22 (0.03) $n = 90$	-0.21 (0.03) $n = 93$	-0.25 (0.03) $n = 90$	-0.20 (0.03) $n = 93$	-0.25 (0.03) $n = 90$
4 to 6 mm	-0.71 (0.05) $n = 92$	-0.86 ^c (0.05) $n = 90$	-0.83 (0.05) $n = 93$	-0.98 ^c (0.05) $n = 90$	-0.86 (0.05) $n = 93$	-1.03 ^c (0.05) $n = 90$
≥ 7 mm	-0.91 (0.10) $n = 78$	-1.38 ^d (0.10) $n = 79$	-1.14 (0.13) $n = 78$	-1.59 ^c (0.13) $n = 79$	-1.17 (0.13) $n = 78$	-1.55 ^c (0.13) $n = 79$

^aNegative change from baseline indicates attachment gain or improvement from baseline.

^bValues represent the least-square means adjusted for investigator and baseline average.

^c $p < 0.05$ versus placebo.

^d $p < 0.01$ versus placebo.

ABBREVIATIONS: cALv = clinical attachment level; PD = probing depth; SDD = subantimicrobial doxycycline.

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TABLE 2. Effect of adjunctive SDD on the mean per-patient average change in PD from baseline (SRP study)^{a,b}

Baseline PD	Mean change (\pm SEM) in PD from baseline (mm)					
	Month 3		Month 6		Month 9	
	Placebo	SDD	Placebo	SDD	Placebo	SDD
0 to 3 mm	-0.03 (0.02) <i>n</i> = 92	-0.12 ^c (0.02) <i>n</i> = 90	-0.07 (0.02) <i>n</i> = 93	-0.16 ^c (0.02) <i>n</i> = 90	-0.05 (0.02) <i>n</i> = 93	-0.16 ^c (0.02) <i>n</i> = 90
4 to 6 mm	-0.60 (0.05) <i>n</i> = 92	-0.82 ^c (0.05) <i>n</i> = 90	-0.68 (0.05) <i>n</i> = 93	-0.91 ^c (0.05) <i>n</i> = 90	-0.69 (0.05) <i>n</i> = 93	-0.95 ^c (0.05) <i>n</i> = 90
≥ 7 mm	-0.93 (0.10) <i>n</i> = 78	-1.55 ^c (0.10) <i>n</i> = 79	-1.14 (0.12) <i>n</i> = 78	-1.75 ^c (0.12) <i>n</i> = 79	-1.20 (0.12) <i>n</i> = 78	-1.68 ^c (0.12) <i>n</i> = 79

^aNegative change from baseline indicates PD decrease or improvement from baseline.^bValues represent the least-square means adjusted for investigator and baseline average.^c*p* < 0.01 versus placebo.

ABBREVIATIONS: PD = probing depth; SDD = subantimicrobial dose doxycycline.

Efficacy Results of SRP Studies

In general, improvements in CALv from baseline were demonstrated for the adjunctive SDD treatment group and the adjunctive placebo group, presumably owing to the course of SRP administered at the baseline visit. However, the mean attachment gains were significantly greater with adjunctive SDD than with adjunctive placebo in tooth sites with mild-to-moderate disease (all *p* < 0.05) and in tooth sites with severe disease (all *p* < 0.05) after 3, 6, and 9 months of treatment. The mean per-patient average change in CALv from baseline for each disease stratum is shown in TABLE 1.

Reductions in PD from baseline also were demonstrated for both treatment groups, presumably owing to the course of SRP administered at baseline. However, the mean reductions in PD were significantly greater with adjunctive SDD than with adjunctive placebo at every time point in tooth sites with mild-to-moderate disease (all *p* < 0.001) and tooth sites with severe disease (all *p* < 0.01) after 3, 6, and 9 months of treatment (TABLE 2).

Adjunctive SDD reduced the incidence of BOP in tooth sites with no disease and in tooth sites with mild-to-moderate disease compared with adjunctive placebo (all *p* < 0.05). Significant treatment differences were demonstrated between adjunctive SDD and adjunctive placebo after 3, 6, and 9 months in tooth sites with no disease (all *p* < 0.05) and after 9 months in tooth sites with mild-to-moderate disease

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($p < 0.05$). In sites with severe disease, a trend favoring adjunctive SDD over adjunctive placebo was noted.

Safety Results of SSDP and SRP Studies

A total of 428 patients (those patients receiving SDD 20 mg twice daily or placebo) were included in the safety analysis. Treatment with adjunctive SDD was well tolerated. The percentage of patients discontinuing treatment with adjunctive SDD due to adverse events of all causes was similar to that for placebo (6% versus 7%, respectively). The nature of adverse events leading to study discontinuation did not differ between the treatment groups.

The most frequent adverse events of all causes are shown in TABLE 3. Adverse events were generally transient and mild to moderate in severity. For both treatment groups, the most commonly reported adverse event was headache, followed by the common cold and flu symptoms. Patients randomized to placebo reported a slightly higher incidence of flu symptoms, toothache, and periodontal abscess than patients randomized to SDD. No clinically meaningful differences in the incidence of adverse events related to the gastrointestinal tract, urogenital tract, or the skin were noted between SDD and placebo. Between the two groups, no clinically meaningful differences in laboratory parameters were demonstrated.

TABLE 3. Incidence of the most common adverse events of all causes ($\geq 5\%$ for either treatment group)

Adverse event	SDD 20 mg bid (n = 213)	Placebo (n = 215)
Headache	26%	26%
Common cold	22%	21%
Flu symptoms	11%	19%
Toothache	7%	13%
Periodontal abscess	4%	10%
Tooth disorder	6%	9%
Nausea	8%	6%
Sinusitis	3%	8%
Injury	5%	8%
Dyspepsia	6%	2%
Sore throat	5%	6%
Joint pain	6%	4%
Diarrhea	6%	4%
Sinus congestion	5%	5%
Coughing	4%	5%

SDD = subantimicrobial dose doxycycline.

Microbiology Results of SSDP and SRP Studies

In general, the results of the studies demonstrate that treatment with adjunctive SDD 20 mg twice daily for up to 12 months did not result in a detrimental shift in the periodontal flora. The use of adjunctive SDD did not result in the colonization or overgrowth of periodontal flora by periodontal or opportunistic pathogens, including yeast and enteric microorganisms. Moreover, treatment with adjunctive SDD did not lead to the emergence of doxycycline resistance or multiantibiotic resistance of the subgingival microflora. No replacement or overgrowth of the periodontal flora by doxycycline-resistant bacteria or yeast was demonstrated.

DISCUSSION

Periodontopathic bacteria and destructive host responses are involved in the initiation and progression of AP. Therefore, the successful long-term management of AP may require an approach to treatment that integrates therapies that address both etiologic components. In the studies described, SDD was used as a systemic adjunct to antimicrobial mechanical interventions in patients with AP. Improvements in periodontal parameters attributable to treatment with SDD likely arise, at least in part, from reductions in the activity of neutrophil collagenase (MMP-8), as shown previously,¹² and confirmed in the Phase II study conducted in patients with AP.

The results of the large-scale, Phase III studies demonstrate that adjunctive SDD improves the efficacy of mechanical interventions routinely used in treating AP in its initial stages. In the combined SSDP studies, treatment with adjunctive SDD resulted in significantly greater gains in attachment than did treatment with adjunctive placebo (all $p < 0.05$). Moreover, improvements in cALv were paralleled by similar improvements in PD; significant reductions in PD were demonstrated in the adjunctive SDD group compared with the adjunctive placebo group (all $p < 0.05$).

Likewise, in the SRP study, significantly greater improvements in cALv and PD were demonstrated with adjunctive SDD than with adjunctive placebo. Improvements from baseline were up to 52% greater for cALv and up to 67% greater for PD with adjunctive SDD than improvements with adjunctive placebo. Furthermore, improvements in clinical parameters were demonstrated with SDD after only 3 months of daily use, and attachment gains and reductions in pocket depth were maintained at 9 months of treatment with SDD.

In all Phase III studies, improvements in BOP were demonstrated with SDD 20 mg twice daily compared with placebo. Improvements in BOP are most likely attributable to improvements in the integrity of the collagen structure at the base of the periodontal pocket rather than to an anti-inflammatory effect.

The present studies also demonstrate that tooth sites with rapidly progressing disease benefit from treatment with adjunctive SDD. It is likely that patients who are highly susceptible to rapidly progressing periodontitis (e.g., those with dysfunctional host responses) and patients with tooth sites refractory to traditional therapies will respond favorably to adjunctive SDD. Evaluating the efficacy and safety of SDD as an adjunct to surgical interventions is a subject of future research.

In general, adjunctive SDD was well tolerated in these studies, with a low incidence of discontinuations due to adverse events. With respect to microbial param-

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ient with adjunctive detrimental shift in the colonization or pathogens, including adjunctive SDD did not affect resistance of the periodontal flora by

involved in the interim management of lesions that address both local and systemic adjunctive improvements in periodontitis, at least in part (2-8), as shown previously with AP.

that adjunctive SDD did not affect AP in its adjunctive SDD result-ant adjunctive plaque levels by similar treatment in the adjunctive all $p < 0.05$).

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ters, treatment with SDD did not alter the dynamics of the subgingival microflora, nor did treatment with SDD lead to the emergence of doxycycline resistance or multi-antibiotic resistance of the subgingival microflora.

To our knowledge, these studies represent the first demonstration of the clinical utility of chronic administration of an MMP inhibitor in a large, Phase III, patient population. These studies provided the basis for the approval of the U.S. Food and Drug Administration to market SDD in the United States under the trade name Periostat®. Periostat is the only MMP inhibitor currently approved for marketing in the United States and is the only product demonstrated to modulate host responses in chronic AP.

When used as an adjunct to mechanical procedures in patients with AP, SDD is a well-tolerated, systemic treatment that significantly improves several indices of periodontal health compared with placebo. SDD, an inhibitor of tissue-destructive MMPs in periodontal tissues, may have clinical utility in the treatment of destructive periodontal diseases.

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Matrix metalloproteinases, tumor necrosis factor and multiple sclerosis: an overview

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Abstract

The matrix metalloproteinases (MMPs) are a family of at least 14 zinc-dependent enzymes which are known to degrade the protein components of extracellular matrix. In addition, MMPs and related enzymes can also process a number of cell surface cytokines, receptors, and other soluble proteins. In particular we have shown that the release of the pro-inflammatory cytokine, tumor necrosis factor- α , from its membrane-bound precursor is an MMP-dependent process. MMPs are expressed by the inflammatory cells which are associated with CNS lesions in animal models of multiple sclerosis (MS) and in tissue from patients with the disease. MMP expression will contribute to the tissue destruction and inflammation in MS. Drugs which inhibit MMP activity are effective in animal models of MS and may prove to be useful therapies in the clinic.

Keywords: Matrix metalloproteinase; Tumor necrosis factor; Multiple sclerosis

1. Metalloproteinases

The metalloproteinases (MPs) are a large family of enzymes which contain a zinc atom in the active site of their catalytic domain (Rawlings and Barrett, 1995). The matrix metalloproteinases (MMPs) are a distinct sub-group of MPs which includes the collagenases, gelatinases, stromelysins, membrane-type metalloproteinases, matrilysin and metalloelastase (Birkedal-Hansen et al., 1993). The MMPs generally have broad, but not necessarily overlapping, substrate specificities. Classically MMP substrates include matrix proteins such as collagen, elastin and fibronectin. However, recent work has extended their targets to include pro-forms of MMPs, enzyme inhibitors and even the cell bound precursors of cytokines and cytokine receptors (reviewed in Table 1). Fig. 1 illustrates the cleavage by MMPs of representative substrates: gelatin (matrix protein), myelin basic protein (major protein of the myelin sheath) pro-tumor necrosis factor- α (TNF) (a

membrane anchored proinflammatory cytokine) and α -1 antitrypsin (an enzyme inhibitor).

MMPs have the potential for massive tissue destruction, and so it is not surprising that their expression and activity is tightly regulated (Docherty et al., 1992). Often, MMPs are only expressed following activation or stimulation of cells. Moreover, MMPs are initially expressed as zymogens which require processing to expose the active catalytic site. This processing step can be achieved by the action of MMPs or other enzymes such as plasmin. Once activated, MMPs are subject to inhibition by tissue inhibitors of metalloproteinases (TIMPs) or by plasma proteins such as α 2-macroglobulin. Despite these controls, excessive MMP production and activation is thought to be a key feature of the pathology of many inflammatory and malignant diseases.

2. Metalloproteinase inhibitors

MMPs have been a target for the pharmaceutical industry for many years and a number of substrate-based pseudopeptide inhibitors have been described (reviewed by

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Table 1
MMP's and their substrates

Interstitial collagenase: (MMP-1)	Collagens I, II, III, VII, VIII, X (Welgus et al., 1990a,b; Sires et al., 1995a,b) (III > I). Gelatin; aggrecan (Fosang et al., 1993) versican (Perides et al., 1995) proteoglycan link protein (Nguyen et al., 1993), casein; α_1 -antitrypsin/ α_1 -proteinase inhibitor (α_1 -AT) (Sires et al., 1994; Desrochers et al., 1991; Mast et al., 1991), α_1 -antichymotrypsin (α_1 -ACHYM) (Desrochers et al., 1991; Mast et al., 1991) α_2 -Macroglobulin; rat α_1 M; pregnancy zone protein; α_1 -inhibitor 3 (α_1 I ₃); ovostatin; entactin (nidogen) (Sires et al., 1993) myelin basic protein (MBP) (Chandler et al., 1995), TNF precursor (Gearing et al., 1994), L-selectin (Preece et al., 1996) MMP-2 (Crabbe et al., 1994a); MMP-9 (Sang et al., 1995)
Neutrophil collagenase: (MMP-8)	Collagens I, II, III, V (Murphy et al., 1982), VII, VIII, X (I > III). Gelatin; aggrecan (Fosang et al., 1993, 1994) α_1 -AT (Desrochers et al., 1992); α_1 -ACHYM (Desrochers et al., 1992); α_2 -antiplasmin (Knäuper et al., 1993a); fibronectin (Tschesche et al., 1992)
Collagenase-3: (MMP-13)	Collagens I, II, III (II > I or III) (Freije et al., 1994; Knäuper et al., 1996a). Gelatin (Knäuper et al., 1996a); α_1 -ACHYM (Knäuper et al., 1996a); plasminogen activator inhibitor 2 (Knäuper et al., 1996a); aggrecan (Fosang et al., 1996); perlecan (Whitelock et al., 1996)
72 kDa gelatinase: (MMP-2)	Collagens I (Aimes and Quigley, 1995), IV (Murphy et al., 1991), V, VII, X (Welgus et al., 1990a), XI, XIV (Sires et al., 1995a). Gelatin; elastin (Murphy et al., 1991; Senior et al., 1991); fibronectin; aggrecan (Fosang et al., 1992); versican (Perides et al., 1995); proteoglycan link protein (Nguyen et al., 1993); MBP (Chandler et al., 1995); A β 1–40 (42) (Roher et al., 1994); A β 10–20 (Miyazaki et al., 1993), APP 695 (LePage et al., 1995); TNF precursor (Gearing et al., 1994); α_1 -AT (Mast et al., 1991); prollysyl oxidase fusion protein (Panchenko et al., 1996) MMP-1 (Crabbe et al., 1994a); MMP-9 (Fridman et al., 1995)
92 kDa gelatinase: MMP-9	Collagens IV (Murphy et al., 1991), V (Murphy et al., 1982), VII, X (Welgus et al., 1990a; Welgus et al., 1990b), XIV (COL 1 domain) (Sires et al., 1995a; Sires et al., 1995b). Gelatin; elastin (Murphy et al., 1991; Senior et al., 1991); aggrecan (Fosang et al., 1992); versican (Perides et al., 1995); proteoglycan link protein (Nguyen et al., 1993); fibronectin (Tschesche et al., 1992); entactin (Sires et al., 1993); α_1 -AT (Desrochers et al., 1992; Sires et al., 1994); MBP (Chandler et al., 1995); TNF precursor (Gearing et al., 1994)
Stromelysin-1: (MMP-3)	Collagens III (weak), IV (Murphy et al., 1991), V, IX, X. Gelatin; aggrecan (Fosang et al., 1991, 1992); versican (Perides et al., 1995); perlecan (Whitelock et al., 1996); proteoglycan link protein (Nguyen et al., 1993); fibronectin; laminin; elastin (Murphy et al., 1991); casein (Windsor et al., 1993); α_1 -AT (Sires et al., 1994; Mast et al., 1991); α_1 -ACHYM (Mast et al., 1991); antithrombin-III (Mast et al., 1991); α_2 M; ovostatin; Substance P; MBP (Chandler et al., 1995) TNF precursor (Gearing et al., 1994); MMP-1 (Murphy et al., 1987); MMP-2/TIMP-2 complex (Miyazaki et al., 1992); MMP-7 (Imai et al., 1995); MMP-8 (Knäuper et al., 1993b); MMP-9 (Shapiro et al., 1995); MMP-13 (Knäuper et al., 1996a)
Stromelysin-2: (MMP-10)	Collagens III (Nicholson et al., 1989), IV (Nicholson et al., 1989; Murphy et al., 1991), V (Nicholson et al., 1989) Gelatin (Nicholson et al., 1989); casein (Windsor et al., 1993; Nicholson et al., 1989); aggrecan (Fosang et al., 1991); elastin (Murphy et al., 1991); proteoglycan link protein (Nguyen et al., 1993); fibronectin (Nicholson et al., 1989); MMP-1 (Windsor et al., 1993); MMP-8 (Knäuper et al., 1996b)
Stromelysin-3: (MMP-11)	Human enzyme: α_1 -AT (Noël et al., 1995); α_2 M (Pei et al., 1994); casein (Pei et al., 1994) mouse enzyme; casein, laminin, fibronectin, gelatin, collagen IV and carboxymethylated transferrin (Murphy et al., 1993)
Matrilysin: (MMP-7)	Collagens IV (Miyazaki et al., 1990; Murphy et al., 1991), X (Sires et al., 1995a,b). Gelatin (Miyazaki et al., 1990); aggrecan (Fosang et al., 1992); proteoglycan link protein (Nguyen et al., 1993); fibronectin and laminin (Miyazaki et al., 1990); insoluble fibronectin fibrils (von Bredow et al., 1995); entactin (Sires et al., 1993); elastin (Murphy et al., 1991); casein (Miyazaki et al., 1990); transferrin (Abramson et al., 1995); MBP (Chandler et al., 1995); α_1 -AT (Sires et al., 1994); TNF precursor (Gearing et al., 1994) MMP-1 (Imai et al., 1995); MMP-2 (Crabbe et al., 1994b); MMP-9 (Imai et al., 1995; Sang et al., 1995)
Macrophage metalloelastase: (MMP-12)	Collagen IV (Chandler et al., 1996). Gelatin (Chandler et al., 1996); elastin and κ -elastin (Shapiro et al., 1993); α_1 -AT (Banda et al., 1987); fibronectin (Chandler et al., 1996); vitronectin (Chandler et al., 1996); laminin (Chandler et al., 1996); proteoglycan monomer (in prep.); TNF precursor (in prep.); MBP (Chandler et al., 1996)
MT-MMP-1: (MMP-14)	Gelatin, casein, κ -elastin, fibronectin, laminin B chain, vitronectin and dermatan sulphate proteoglycan (Pei and Weiss, 1996) MMP-2 (Sato et al., 1994)
MT-MMP-2: (MMP-15)	?
MT-MMP-3: (MMP-16)	MMP-2 (Takino et al., 1995)
MT-MMP-4: (MMP-17)	?

Birkedal-Hansen et al., 1993 applies unless otherwise stated.

Documented activations of other MMPs are shown but self-cleavage (auto-activation) events are not.

Beckett et al., 1996). MMP inhibitors have shown efficacy in models of cancer, acute and chronic inflammatory diseases. The most advanced clinical studies to date have been in corneal ulceration with the Glycomed compound Galardin_{TM} (given by injection), in malignant pleural effusion and ascites with the British Biotech compound Batimastat (given by injection) and in a variety of solid tumors with the British Biotech compound Marimastat (given orally). With the recognition that TNF processing was dependent on an MMP-like enzyme, we have also pro-

duced a number of compounds which combine both potent MMP and TNF inhibition (TMIs) (Gearing et al., 1994).

3. Relationship of MMPs to the cytokine convertase/sheddase

It is not yet clear to what extent MMPs participate in the release of TNF or of other shed membrane molecules in vivo. There is emerging evidence that the shedding

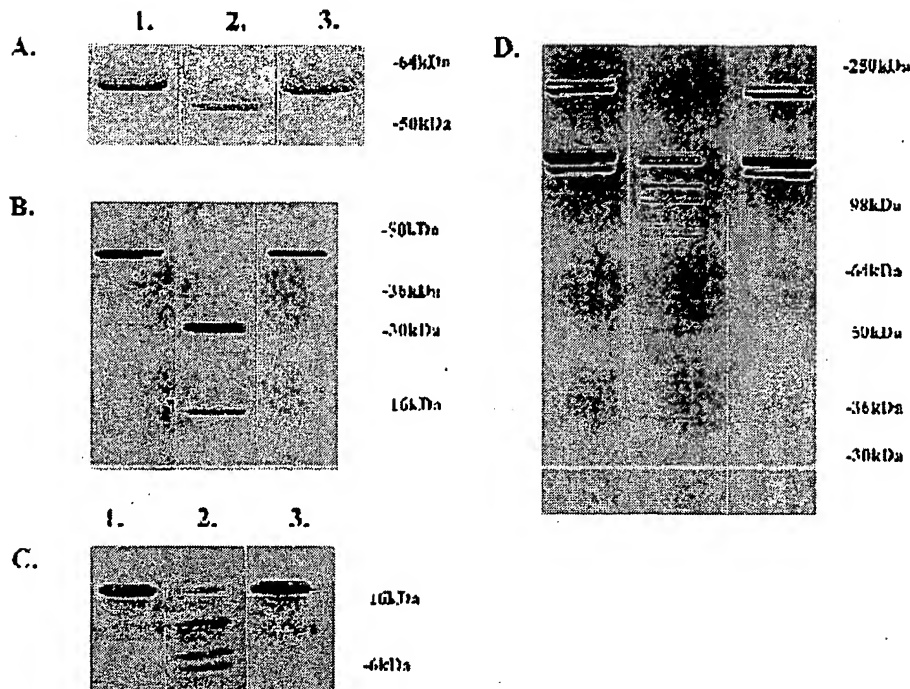


Fig. 1. Cleavage of protein substrates by MMPs. Protein substrates and MMPs were incubated at 37°C for: (A) 1 h with α 1-antitrypsin and matrilysin; (B) 2 h with glutathione s-transferase-linked pro-TNF fusion protein and macrophage metalloelastase; (C) 17 h with bovine myelin basic protein and 72 kDa gelatinase; (D) 20 h with type-1 gelatin and macrophage metalloelastase. Proteolysis was followed by SDS PAGE with Coomassie blue staining. In each case track 1 contained substrate alone, track 2 substrate plus MMP and track 3 substrate, MMP plus 10 μ M of BB-2116, a broad-spectrum MMP inhibitor. (Methodology in Chandler et al., 1995). In each case the substrate was cleaved by the MMP and the cleavage prevented by the MMP inhibitor.

reaction can be mediated by novel metalloproteinases. Although we have shown that many of our MMP inhibitors also prevent the cellular processing of pro-TNF (Gearing et al., 1994), detailed analysis of the inhibitory potency of different compounds suggest that the TNF converting enzyme may be pharmacologically distinct from the classical MMPs (A. Drummond, submitted for publication). The number of cell surface molecules whose shedding is prevented by MMP inhibitors now includes several cytokines (TNF- α , Fas Ligand, TGF- α), cytokine receptors (TNFR1, TNFR2, IL6R- α), adhesion molecules (L-selectin) and others (β -amyloid precursor protein) (Gearing et al., 1994; Arribas et al., 1996; Crowe et al., 1994; Preece et al., 1996; Tanaka et al., 1996). Arribas et al. have recently described a number of mutant CHO cell lines which are defective in the shedding of a range of cell surface molecules including TGF α , IL6 receptor α , L-selectin and β amyloid precursor protein. They have also shown that the shedding reaction in wild-type CHO cells is blocked by an MMP inhibitor (Arribas et al., 1996). A group at Glaxo Wellcome has suggested that the TNF converting enzyme may be a member of the repolysin or adamalysin family of metalloproteinases (Moss et al., 1997). These enzymes have a catalytic domain which is similar to the MMPs but also contain a disintegrin and EGF-like domain (Rawlings and Barrett, 1995; Wolfsberg et al., 1995). The relationship of the shedding mutation

described by Arribas to the TNF converting enzyme remains to be established.

Until we know the identity of the converting enzyme(s) or 'shedase' and until specific inhibitors or knock-out mice are available, it will be difficult to determine the relative contributions of known MMPs to the shedding reactions. At present, however, it seems likely that under pathological conditions where local levels of MMPs are high they could contribute to the release of cell surface cytokines and receptors.

4. Demyelinating neuroinflammatory disease: the case for treatment with a TMI

Multiple sclerosis is a chronic disabling disease of the central nervous system (CNS). The disease is characterised by the presence of demyelinated plaques or lesions, which early in their development have a disrupted blood brain barrier causing leakage of plasma proteins (Calder et al., 1989). These plaques contain inflammatory cells (T lymphocytes and monocytes) in addition to activated glial cells. It is thought that an initial influx of inflammatory cells drives the subsequent development of the lesions. Indeed, in animal models of MS, agents which prevent T cell or macrophage infiltration or activation are effective in reducing disease severity. The destructive nature of the MS

lesions led to an early interest in proteases and mediators of tissue breakdown (Hallpike and Adams, 1969; Cuzner et al., 1978; Inuzuka et al., 1987) which recently has focused on the role of matrix metalloproteinases.

4.1. Evidence for MMP expression in MS

The cellular components of an MS plaque, including activated T cells, macrophages, microglia and astrocytes, are capable of expressing a wide range of MMPs *in vitro* (Apodaca et al., 1990; Conca et al., 1994; Welgus et al., 1990a,b; Yamada et al., 1995a,b; Wells et al., 1996). 92 and 72 kDa gelatinases, stromelysin-1 and collagenase have also been detected either in the plaques or CSF of patients with MS (Gijbels et al., 1992; Steinman and Gijbels, 1994; Maeda et al., 1996). We have also demonstrated elevated levels of 92 kDa gelatinase in the serum of patients undergoing a relapse of MS (Miller et al., 1996). Indirect evidence for expression of TNF converting enzyme activation is provided by the fact that TNF is elevated in the blood and CSF of patients with MS during clinical relapse (see below).

4.2. Consequences of MMP expression in the CNS

There is good evidence that MMP expression in the CNS can contribute to the pathology observed in MS. Injection or induction of MMPs in the brains of rats causes breakdown of the blood–brain barrier (BBB) and tissue destruction (Rosenberg et al., 1992a,b and Fig. 2). There is also evidence that lymphocytes may use MMPs for transmigration through vascular endothelium (Leppert et al., 1995). MMPs have been shown to degrade myelin basic protein, the major protein component, of the myelin sheath which is destroyed in MS (Gijbels et al., 1993; Chandler et al., 1995 and Fig. 1). The processing of MBP has also been shown to liberate immunogenic peptides which may serve to propagate the autoimmune response which drives MS (Opdenakker and Van Damme, 1994).

A further consequence of MMP expression is the cleavage of membrane bound molecules including TNF- α . TNF has been found in blood and CSF of patients with MS (Selmaj et al., 1991; Benvenuto et al., 1991; Sharief and Thompson, 1992). TNF is a potent proinflammatory cytokine which stimulates the expression of other inflammatory cytokines, adhesion molecules and MMPs, and has been claimed to be directly toxic for oligodendrocytes. In animal models of MS TNF inhibitors are effective in reducing disease severity (Sommer et al., 1995; Baker et al., 1994).

Thus expression of MMPs in the CNS can mediate leucocyte recruitment, blood–brain barrier leakage, myelin destruction and can perpetuate the immunoinflammatory response by generating immunogenic peptides and releasing the proinflammatory cytokine TNF (Fig. 3).

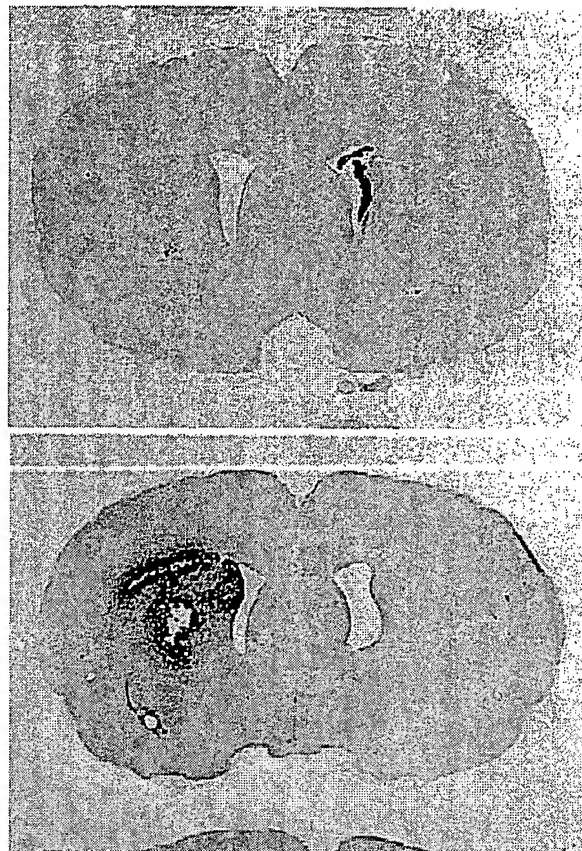


Fig. 2. The effect of intracerebral injection of 72 kDa gelatinase. Stereotactic injections of 1 μ g 72 kDa gelatinase were given into the striatum of adult Lewis rats. 24 h later the rats were injected i.v. with horse radish peroxidase (HRP), killed 20 min later and perfusion fixed. 50 μ m coronal sections were taken and stained with the Hanker–Yates method (Perry and Linden, 1982) to reveal HRP within the tissue. In the buffer injected controls (top panel) no leakage of HRP through the BBB was observed. In the 72 kDa gelatinase injected animals (lower panel) HRP had leaked extensively indicating the BBB had been disrupted.

4.3. Effects of MMP inhibitors in models of MS

Two broad spectrum MMP inhibitors, GM6001 and RO 31-9790, have been shown to reduce disease in acute experimental autoimmune encephalomyelitis (EAE) in the rat (Gijbels et al., 1994; Hewson et al., 1995). We have also shown that BB-1101, a broad spectrum MMP inhibitor which is also a potent inhibitor of TNF production *in vivo* (Corkill et al., 1995), is also effective in rat EAE reducing disease severity and weight loss (Clements, 1997). BB-1101 has also been tested in a recently described delayed-type hypersensitivity (DTH) model of MS (Matyszak and Perry, 1995). In this model, an immune-mediated inflammatory response against a non-CNS antigen is established in the brain. This response generates a focal, demyelinated lesion which resembles the plaques in MS. BB-1101 inhibits both the inflammation and demyelination in this model (Matyszak and Perry, 1996). Given the multiple mechanisms by which MMPs could contribute

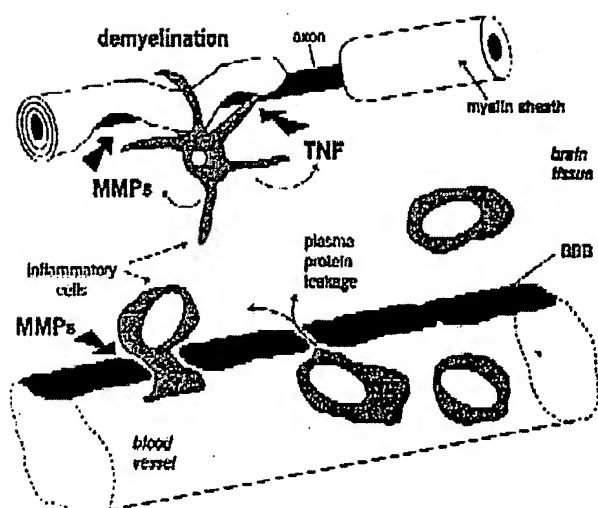


Fig. 3. Contribution of MMPs to the pathology of MS. MMPs are produced by invading T cells and macrophages as well as resident glial cells. MMPs can disrupt the BBB allowing inflammatory cells and plasma proteins such as antibody and complement to enter the lesion. MMPs also mediate the destruction of extracellular matrix and myelin components. Soluble TNF generated by MMP action is toxic for oligodendrocytes, promotes the production of inflammatory mediators and further MMP expression and can mediate BBB damage.

to the pathology of MS, it is not obvious whether MMPs, TNF (or other shed molecules) or both are the principle targets for MMP inhibitors. We are currently profiling the expression of MMPs in these animal models and in MS tissues and body fluids to determine which enzymes could be key to the pathology.

5. Summary

MMPs are potent mediators of tissue destruction in autoimmune disease. MMPs also contribute to the release or shedding of cell surface molecules such as TNF- α . There is considerable evidence that MMPs are expressed in the lesions of MS where they contribute to leucocyte invasion, BBB breakdown, myelin destruction and TNF release. Compounds which inhibit MMP activity and TNF release are active in animal models of MS. In view of this, clinical trials of a broad spectrum MMP inhibitor should be considered.

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Inhibitors of tumor necrosis factor- α : Promising agents for the treatment of multiple sclerosis?

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Tumor necrosis factor (TNF)- α is a critical inflammatory mediator of experimental autoimmune encephalomyelitis and multiple sclerosis, and may therefore be a useful target for immunotherapy. Therapeutic strategies aimed at TNF include pharmacological inhibitors of TNF synthesis and/or processing and biological inhibitors of TNF effects. Several anti-TNF agents are currently being tested in multiple sclerosis in pilot clinical trials.

Keywords: multiple sclerosis; tumor necrosis factor- α ; immunotherapy

Basic properties of TNF and TNF receptors

Cytokines are soluble mediators of cell-to-cell communication.¹ A characteristic feature of all cytokines is their functional redundancy and pleiotropy.¹ Tumor necrosis factor (TNF)- α is an especially pleiotropic cytokine produced by many cell types. TNF- α exerts diverse effects on different cells and tissues (Table 1). As a mediator of inflammation, TNF- α stimulates the release of interleukin-1, many other cytokines and all metabolites of arachidonic acid.^{2,3} Through its capacity to influence leukocyte traffic by inducing or enhancing the expression of adhesion molecules on endothelial cells, TNF- α facilitates the local accumulation of macrophages, neutrophils and lymphocytes. In association with interferon- γ , TNF- α induces or enhances the production of reactive oxygen and NO derivatives, mediators that protect against microbial and parasitic invaders but may also damage the host. Since TNF- α can induce its own transcription, special – as yet incompletely understood – control mechanisms are required.

There are two types of TNF receptor (TNF-R₁ and TNF-R₂), which are also recognized by the closely related cytokine lymphotoxin.^{4,5-10} Both the ligand and the TNF receptors exist in a fixed, membrane-bound and a shed, soluble form. At least one type of TNF receptor is expressed in most if not all cells. The TNF receptors belong to a receptor family that includes the Fas receptor CD95 (which mediates apoptosis), nerve growth factor receptor CD27 (which provides a costimulatory signal for T cell proliferation) and CD40 (a critical factor of B cell growth and differentiation). It appears that these receptors share the ability to regulate cell growth and death in different tissues.

Binding of the homotrimeric ligand TNF- α links three receptors together into a cluster. Ligand-induced crosslinking brings the cytoplasmic regions of the receptors together, initiating a self-assembly process in which additional cytoplasmic proteins bind to form a

complex with catalytic activity. There is evidence that the TNF receptors use different signaling pathways, including the sphingomyelin pathway, to mediate biological responses, but many aspects of TNF-mediated signal transduction are still unknown.^{4,5-10}

Role of TNF- α in experimental autoimmune encephalomyelitis and multiple sclerosis (Table 2)

TNF- α is expressed in MS lesions, where it is associated with CD3+ lymphocytes, microglia cells and astrocytes.^{11,12} Furthermore, TNF- α and lymphotoxin are cytotoxic for oligodendrocytes *in vitro*.¹³⁻¹⁵ In experimental autoimmune encephalomyelitis (EAE), secretion of lymphotoxin and TNF- α by T cells specific for myelin basic protein (MBP) correlates with the encephalitogenic potential of the T cells.¹⁶ Treatment with an anti-TNF monoclonal antibody reduced the severity of EAE transferred with MBP-specific encephalitogenic T cells.

Table 1 Tumor Necrosis Factor (TNF)- α : Basic Facts

- 157 amino acids, homotrimer
- Production by macrophages, T cells and many other cells
- Two receptor types (TNF-R₁ and TNF-R₂), widely distributed
- Activates inflammatory cells; regulates cell growth, differentiation and many other cell functions; has prominent cytotoxic effects

Table 2 Role of TNF- α in Multiple Sclerosis

- Expression in MS lesions
- *In vitro* cytotoxicity for oligodendrocytes
- (Loose?) correlation of TNF- α concentrations/expression with clinical activity
- Increased production of TNF by T cells from HLA-DR2+ individuals

Table 3 Strategies for TNF Inhibition

- Pharmacological inhibitors of TNF synthesis or processing: Pentoxifylline, thalidomide, rolipram, pyridinyl-imidazole protein kinase inhibitors, metalloproteinase inhibitors, glucocorticosteroids
- Biological inhibitors of TNF effects: Anti-TNF antibodies; soluble TNF receptor constructs

phallitogenic T cells.^{17,18} Furthermore, several studies indicated that in MS patients, there is a correlation between TNF levels in blood, serum or culture supernatant and the clinical course.¹⁹⁻²¹ Finally, the well-established genetic association of multiple sclerosis with HLA-DR2 can be at least partly explained by a propensity of HLA-DR2+ T cells to produce increased amounts of TNF- α and lymphotoxin.²²

Inhibitors of TNF synthesis or processing

Different strategies for TNF inhibition are shown in Table 3. The pharmacological inhibitors of TNF synthesis pentoxifylline and thalidomide are tested in pilot clinical trials.²³ The antidepressant rolipram, a selective inhibitor of phosphodiesterase type IV,²⁴ has shown promising effects on EAE in rats and common marmoset monkeys.^{25,26} However, this agent is currently unavailable for clinical trials in multiple sclerosis. A series of pyridinyl-imidazole compounds inhibit the production of interleukin-1 and TNF- α at the translational level.²⁷ This class of cytokine inhibitors may provide new therapeutic candidates in the future.²⁸

Interestingly, recent evidence suggests that corticosteroids, which are widely used for the treatment of acute exacerbations of multiple sclerosis, also exert at least part of their immunosuppressive actions by inhibition of TNF- α .^{29,30} Corticosteroids stimulate production of I κ B α , a protein that holds the transcription factor NF- κ B in inactive form in the cytoplasm.^{31,32} NF- κ B, a major regulator of many cytokine and cell adhesion genes, is critically involved in the TNF signaling pathway.⁶

A different group of agents aims at the processing of the 233-amino-acid membrane-bound TNF- α precursor into the mature, 157-aminoacid cytokine. This process is dependent on matrix metalloproteinase enzymes, and inhibitors of matrix metalloproteinases are very potent inhibitors of TNF processing (but not synthesis) *in vitro*.³³⁻³⁶

Biological inhibitors of TNF effects

Biological inhibitors of TNF include monoclonal antibodies against TNF- α and soluble TNF receptor constructs (Tables 3 and 4). Several of these agents have shown positive effects in EAE.^{17,27,37} Furthermore, biological inhibitors of TNF are being tested in rheumatoid arthritis (Table 4).^{38,39} In multiple sclerosis, a clinical trial of soluble dimeric TNFR₂ coupled to a human IgG1 Fc framework has recently been initiated.

Inhibitors of TNF- α
R Hohlfield

Table 4 Biological TNF inhibitors in rheumatoid arthritis

Anti-TNF monoclonal antibodies

- Human/murine IgG1 κ chimeric neutralizing Ab cA2 (Centocor): Positive results in randomized double-blind placebo-controlled trial of a single infusion (1 or 10 mg/kg) of cA2 in 73 patients with active RA (39). Repeated therapy with mAb cA2 (2-4 cycles) also successful, but antibodies to the murine portion of cA2 eventually detected in 50% of patients (40)
- Humanized anti-TNF- α IgG4 mAb CDP571 (CellTech): Beneficial effect in preliminary (dose escalation) study

Soluble TNF receptors (sTNFR)

- Soluble dimeric p-60 TNFR coupled to a human IgG1 Fc framework (Immunex): Beneficial effect in preliminary studies
- Soluble dimeric p-60 TNFR coupled to a human IgG1 Fc framework (Roche)

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apo E receptors following conversion within the body to chylomicron remnants. These receptors, present on the cell surface of hepatocytes, internalize the ligand with eventual degradation within lysosomes¹². Because there is no direct linkage or modification of the apo E ligand in this strategy, large amounts of drug can theoretically be incorporated without altering the targeting specificity. In this respect, the report is conceptually similar to liposomes into which ligands and agents have been incorporated¹³, though the size and specificity of liposomes has generally been difficult to control. The use of a recombinant apo E can potentially provide a large-scale, uniform source of ligand for therapeutic purposes.

Despite the virtues of the lipoprotein-based systems, there are limitations. The drug must be either intrinsically lipophilic or capable of being modified to possess this property. The modified pro-drug must be able to be converted to its active form within the target cell. Because the intracellular pathway is presumably degradative, the agent must be able to withstand exposure to powerful enzymes and yet remain active. The release of the pro-drug must also permit ac-

cumulation of sufficiently high levels of active drug within the cell. Finally, this delivery system is not likely to have ameliorative effects on any intrinsic cytotoxicity that the agent itself might possess.

Apart from these limitations, the article by Rensen *et al.* provides a practical demonstration of lipoprotein-based drug targeting vehicles. It also illustrates the power of mimicry of natural systems to achieve biological — and potentially clinical — objectives.

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Multiple sclerosis: TNF revisited, with promise

The antidepressant drug rolipram inhibits tumor necrosis factor- α . This may make it an effective treatment for multiple sclerosis (pages 244–248).

CEDRIC S. RAINE

The immune system in health can be likened to an orchestra with different musicians and instruments contributing to a harmonious symphony, or in biologic terms, immunologic homeostasis. The immune system in disease, however, is an orchestra in disarray with one or more lead members out of key. This is particularly true in those instances when the target of the immune response is one of the body's own proteins — a time when autoimmunity becomes the operative term and when normal regulatory mechanisms sometimes do more harm than good. With this analogy in mind, the present commentary addresses an immunologic phenomenon featured in an interesting article in the present issue of *Nature Medicine* by Norbert Sommer and colleagues in Roland Martin's laboratory in Tübingen¹. These authors describe

experiments in an autoimmune disease in rats used as a model for multiple sclerosis (MS), which confirm that administration of a pharmacologic preparation with a known propensity to down regulate and inhibit at least one key player in the orchestra, the cytokine tumor necrosis factor- α (TNF- α), is capable of disrupting the anticipated course of events and silencing the disease.

While somewhat melodramatic and overstated, the above description highlights an important and topical area of biologic research with wide-ranging, disease-relevant ramifications. Immunologic attack against tissue-specific self-antigens might be abrogated by the

administration of broad-acting soluble mediators of the immune system, a concept which has been tested in most conditions of suspected or proven autoimmune etiology. Moreover, as members of the growing family of these soluble mediators — collectively referred to as cytokines — were characterized, it became apparent that some serve to promote the immunologic response (proinflammatory cytokines), while others modulate or suppress it (regulatory cytokines)². Cytokines operate in concert, mediating much of the intercellular signalling required for an integrated response to a variety of external stimuli. Cytokines are efficient and potent mediators that interact with specific, high-affinity soluble and membrane-bound cellular receptors. They are inducible, oft-times short-lived proteins, and the ability to generate them

in large amounts in recombinant form has provided much meat for the biotechnology community.

As stated, the therapeutic application of an inhibitor of one of these cytokines — the proinflammatory molecule $\text{TNF-}\alpha$ — to an animal model of MS, experimental autoimmune encephalomyelitis (EAE) in rats, forms the basis of this *News & Views*. The immediate goal of the work by Sommer *et al.* was to prevent clinical disease (paralysis) by blocking inflammation from occurring in the central nervous system (CNS), thus preventing demyelination. Myelin, the insulating membrane of nerve fibres, is essential for normal conduction. It is a highly immunogenic membrane and one unique to nervous tissue⁴. Its loss from axons leaves the nerve fiber demyelinated and functionally impotent due to the dispersal of its sodium and potassium channels.

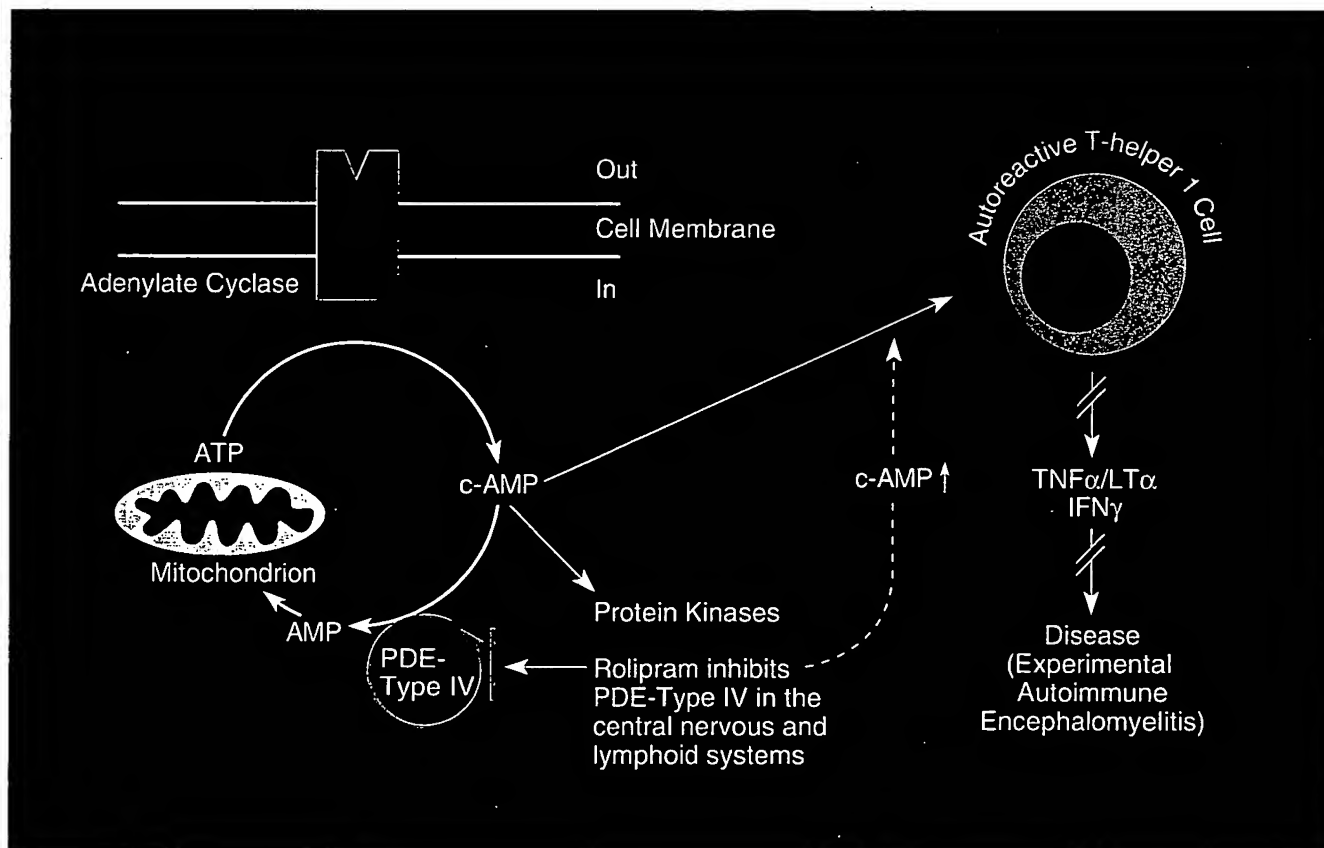
Multiple sclerosis, the paradigm of demyelinating diseases, is a neurologic

condition in which myelin is destroyed by an inflammatory process of unknown aetiology⁵. It is usually a chronic progressive or chronic relapsing condition with an unpredictable course spanning several decades. Evaluation of the efficacy of compounds like cytokines and their inhibitors upon the course or nature of the inflammatory response in the CNS of the MS subject is difficult because of the innate variability in clinical and pathologic manifestations from patient to patient — hence the need for an animal model in which the hallmark lesion, inflammatory demyelination, is the major feature³.

What might not be readily appreciated, is that in diseases as diverse as MS, rheumatoid arthritis, Crohn's disease, systemic lupus erythematosus, some forms of diabetes, and other diseases involving a selective immunologic attack upon a particular organ or system, common mechanisms may pre-

vail. In other words, following the recognition of the targeted self-antigen(s) in each disease (presumably by antigen-specific T-cells), the selective damage to the respective tissue may involve the same cascade of soluble immune mediators with no particular organ specificity. It was with this in mind that some early work on the suppression of autoimmune demyelination, like that of Celia Brosnan and colleagues⁶, focused on the systemic inhibition of proteolytic enzyme activity by macrophages. This approach proved effective in a rat model of EAE using inhibitors of plasminogen activator and neutral proteinases. Precisely what else was being affected by the enzyme inhibitors remains speculative, but today one could quite easily invoke a role for cytokines.

The current report by Sommer *et al.*¹, shows that intraperitoneal injections of the antidepressant rolipram are capable of preventing, delaying and reducing



Schematic presentation of the effects of the antidepressant rolipram on phosphodiesterase (PDE) type IV and T-helper 1-mediated autoimmune responses. Rolipram specifically inhibits the PDE type IV expressed in lymphoid and central nervous system cells. PDE inhibition raises the cyclic AMP level (left), which in turn interferes with activation and lymphokine secretion of autoreactive T-helper cells (right). Tumour necrosis factors ($\text{TNF-}\alpha/\text{LT-}\alpha$) and interferon- γ ($\text{IFN-}\gamma$) secreted by these cells are involved in the pathogenesis of autoimmune demyelinating diseases such as experimental autoimmune encephalomyelitis (EAE). PDE type IV inhibition by rolipram blocks these effector mechanisms and can be used to treat EAE. [Courtesy of Roland Martin, National Institutes of Health, Bethesda, Maryland.]

the clinical severity of acute EAE in rats. The clinical findings were dose-dependent and parallel studies on the inhibition of TNF- α production *in vitro* showed the effect to be stereospecific inasmuch as the negative enantiomer was 55 times more effective than the positive enantiomer. While it was speculated that the action of the drug was related to its effect upon cytokine levels, particularly TNF- α (and to a lesser extent, IFN- γ), it should be noted that rolipram, a wide-acting compound and a selective type IV phosphodiesterase inhibitor, also affects metalloprotease and NO levels. These pathways may merit further investigation. Critics of the present report may also feel that the levels of the same cytokines (and perhaps some enzymes) within the CNS should have been taken into consideration, because positive correlation might have bolstered their putative involvement in this autoimmune disease.

The findings of Sommer *et al.* provide further confirmation of a specific role for TNF- α during CNS demyelination. In 1988 it was shown by Kris Selmaj and myself⁶ that recombinant TNF- α (obtained from Marc Feldmann in London) induced selective destruction of oligodendrocytes and myelin in culture. The selective cytokine-mediated demise *in vitro* of oligodendrocytes — cells which produce and maintain CNS myelin — was particularly relevant, because in MS, oligodendrocyte depletion from demyelinated plaques figures prominently and remains an enigma³. Subsequent work on dissociated mixed glial cell cultures^{7,8} supported the selective cytolytic effect of TNF- α upon oligodendrocytes. They also showed that TNF- α exerts a stimulatory effect upon astrocytes, the cells responsible for scarring in MS.

The tissue culture approach assumed clinical relevance with the work of Nancy Ruddle — who along with Byron Waksman is a pioneer in the TNF/lymphotoxin (LT) field⁹ — and her colleagues at Yale. They showed that a monoclonal antibody to TNF- α and LT could prevent acute EAE in mice¹⁰. This report was confirmed and extended shortly thereafter with a study from my laboratory using a chronic relapsing form of EAE in the mouse and a poly-

clonal antibody to TNF- α ¹¹. More pieces were fit into the puzzle when TNF- α was localized in MS plaques,^{12,13} and when circulating levels of TNF- α and its soluble receptor were found in the CSF of MS patients^{14,15}.

The association between TNF- α levels and the expression of EAE using a TNF- α inhibitor was first approached in a study similar to that of Sommer *et al.*¹ in which phosphatidylserine (PS), a natural membrane phospholipid and a potent inhibitor of endotoxin-induced

In diseases as diverse as MS, rheumatoid arthritis, Crohn's disease, systemic lupus erythematosus, some forms of diabetes, and other diseases involving selective immunologic attack on a particular organ or system, common mechanisms may prevail.

TNF- α production, was applied to the mouse model of chronic relapsing EAE and shown to reduce significantly clinical and pathologic signs of disease¹⁶. In both antibody-treated¹¹ and PS-treated¹⁶ mice, the effect of the treatment regimens was short-lived; when treatment ceased after one to two weeks, signs of disease appeared. However, prolonged treatment with PS had a lasting effect¹⁶. More recently, it was shown that soluble TNF receptor can also prevent EAE^{17,18}.

The work of Sommer *et al.*¹ adds further weight to a TNF- α approach in MS. As is always the case, however, some points need to be clarified. In their final paragraph, the authors push for the testing of Rolipram in MS and mention *en passant* that preliminary data on chronic-relapsing EAE in the mouse lend support to the effectiveness of this drug.

Interestingly, waiting in the wings is a paper currently in press in *The Proceedings of the National Academy of Sciences*¹⁹ by Claude Genain and colleagues in Stephen Hauser's laboratory, which also describes tests of rolipram in EAE. More significantly, perhaps, is that this group is one of a very few working on EAE in non-human primates. These investigators have now applied their model of EAE in mar-

mosets²⁰ to the testing of this TNF- α inhibitor. The results have revealed no signs of EAE in three animals receiving rolipram every other day beginning one week after immunization for EAE for a total of 46 days. Protection was sustained and MRI showed no abnormality. Placebo and untreated groups developed the usual manifestations of EAE. The demonstration of a successful effect by rolipram in a model of primate EAE, a model with many similarities to human MS, lends more weight to the TNF approach and holds promise for MS.

In closing it is only fair to mention that TNF- α is not the only cytokine under study in MS-related research. Reports exist in the literature showing a role for IL-1²¹, IL-4²², IL-10²³, IL-12²⁴, IFN- γ ²⁵, and TGF- β ^{26,27,28}. This commentary highlights the TNF- α approach in MS in the context of the article appearing in this issue of *Nature Medicine*. Nevertheless, although it is well recognized

that few, if any, cytokines function unilaterally (synergism and synchrony between members being the norm in nature), the volume of evidence documenting a distinct role for TNF- α in MS is quite impressive, and is currently greater than for any other cytokine. Therefore, it appears that TNF- α may have taken the lead. So pass the baton to TNF- α , have the orchestra strike-up, and let us sit back and enjoy the concert. Music, Maestro, please!

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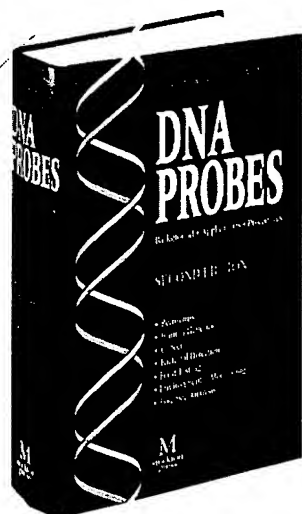
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The potential use of MMP inhibitors to treat CNS diseases

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Review

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The matrix metalloproteinases (MMPs) are considered to be the physiological mediators of extracellular matrix remodelling. MMPs are involved in a variety of functions and in the nervous system, these include angiogenesis and the extension of neuronal growth cones during development. However, it has become increasingly evident that the aberrant expression of MMPs in the nervous system contributes to diseases that include among others, multiple sclerosis, malignant gliomas, Alzheimer's disease and stroke. This review highlights the evidence that MMPs are involved in diseases of the nervous system, and provides information for the potential beneficial use of MMP inhibitors in NS disorders. However, the application of MMP inhibitors to treat CNS diseases must be balanced carefully against the beneficial roles normally played by MMPs in CNS physiology or recovery.

Keywords: *Alzheimer's disease, cerebrovascular disorders, gliomas, matrix metalloproteinases, multiple sclerosis, myelination, stroke*

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1. Introduction to MMPs

Matrix metalloproteinases (MMPs, matrixins) are considered the physiological mediators of degradation, or remodelling, of the extracellular matrix (ECM). They are implicated in the modulation of cell-ECM interactions that govern processes as diverse as cellular differentiation, migration and apoptosis, or the regulation of growth factor activity [1]. Collectively, the MMPs degrade most, if not all, protein components of the ECM. Some postulated physiological functions mediated by MMPs are shown in **Table 1**.

1.1 MMP family members

There are at least 18 MMP members, divided into four classes based on structure: collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs) (**Figure 1**). Each MMP member is the product of a different gene. However, sequence conservation in MMP genes and the observation that some MMPs contain more related domains than others, suggest that the various family members may have evolved from a single ancestral gene by gene duplication and exon shuffling. All MMPs have a common propeptide region at the N-terminus, in which an invariant cysteine residue ligates back to zinc at the catalytic domain, thus maintaining the enzyme in its inactive state. The C-terminus domain, with a high level of homology to members of the hemopexin family, confers substrate binding and is present in all MMPs except matrilysin. Additional

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Table 1: Physiologic roles of MMPs

Normal processes	Pathologic processes
Ovulation	Cancer metastasis
Blastocyst implantation	Rheumatoid arthritis
Embryogenesis	Periodontal disease
Bone growth/remodelling	Alzheimer's disease
Angiogenesis	Gastric ulcer and liver cirrhosis
Neuronal migration and neurite extension	Atherosclerosis
Wound healing	Fibrotic lung disease
ECM modelling	Inflammation and multiple sclerosis

structures, such as the presence of fibronectin Type II-like modules which bind native basement membrane Type IV and V collagens, elastin, and all denatured collagens, are present in gelatinases and confer further substrate binding capacity. C- and N-terminus domains are connected by a hinge region which is short in collagenases and long in other MMPs. The MT-MMPs have an additional membrane domain at the C-terminus and are transmembranous proteins. Present in all MMPs, there is also a short signal sequence in the extreme N-terminus.

Specificity for substrates is useful to subclassify MMPs, but there is much overlap. Nonetheless, collagenases are thought to degrade fibrillar collagens, stromelysins prefer proteoglycans and glycoproteins, and gelatinases are particularly potent in the degradation of non-fibrillar and denatured collagens. Besides facilitating the activation of some MMPs (see section 1.2), the transmembranous MT-MMPs also have proteolytic activity against ECM components, including gelatins [2,3].

1.2 Control of MMP activity

Being proteolytic enzymes, MMPs have the potential for massive tissue destruction. Therefore, the activity of MMPs has to be controlled; this is achieved on three levels (Figure 2) [4]. First, all MMPs are expressed as inactive zymogens and require processing to expose the active catalytic site. In this regard (reviewed in [5]), activating factors, including plasmin and other MMPs, first disrupt the cysteine zinc interaction. The partially active intermediate is then subjected to removal of the entire propeptide region, by proteinases including other MMPs, for full activation. Zymogens can also be activated by non-proteolytic compounds such as SH reactive reagents (4-aminophenylmercuric acetate,

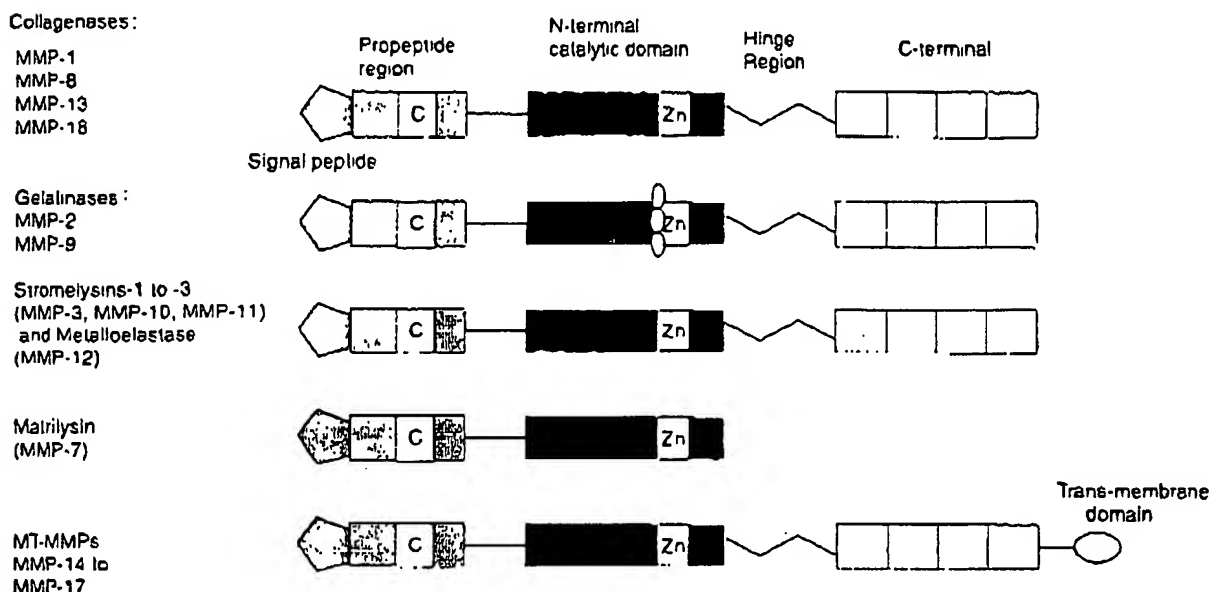
AMPA), denaturants (urea, SDS) and by heat treatment.

A second means to control MMP activity involves gene transcription, and most MMPs are expressed only following the activation/stimulation of cells. The transcription of MMPs is effected by a number of agents including phorbol esters, growth factors and oncogene products. In most cases, these factors induce the expression of the c-fos and c-jun proto-oncogenes, which form a heterodimer that binds to promoter sequences of DNA called the phorbol responsive element (TRE), or the activator protein-1 (AP-1) binding site, to affect gene transcription. The AP-1 element is notably absent in the promoter region of MMP-2 and this MMP is normally expressed at a constitutive rather than inducible level. Other transcriptional binding elements (e.g., PEA-3, Sp1, NFkB), are also present in several MMPs [6], and these can influence the activity of the AP-1 transcriptional element. Cell-ECM and cell-cell interactions also affect MMP transcription [7] and the engagement of various integrin receptors by their ligands induces MMP gene expression (reviewed in [8]).

A third means to control MMP activity is by the interaction of active MMPs with tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs are currently known and these bind the catalytic active site of MMPs to cause inactivation [9,10].

Interestingly, it was noted that the cell surface activation of proMMP-2 by MT1-MMP requires the interaction of TIMP-2 [11]. Subsequently, it was shown that MT1-MMP may function as a receptor for TIMP-2 (but not TIMP-1) and/or the proMMP-2/TIMP-2 complex, facilitating the proteolytic activation of proMMP-2 by an adjacent MT1-MMP (reviewed in [12]). The membrane requirement of proMMP-2 is also

Figure 1: Structure of the subgroups of MMPs.



due to the co-localisation of MMP-2 with $\alpha\text{V}\beta 3$ on cell surfaces, as is the case for invasive cells [13]. Similarly, proMMP-9 can also form a complex with TIMP-1 (and proMMP-2 with TIMP-3), to facilitate its activation.

Besides the mode of interaction of specific TIMPs with particular MMPs (generally N-N interactions for enzyme activity inhibition, and C-C interactions for pro-enzyme activation), the role of TIMPs in both the activation and deactivation of MMPs appears to be concentration-dependent, in that low to moderate levels of TIMPs facilitate pro-enzyme activation, while higher TIMP levels will lead to inhibition of the active protease.

Other less well characterised tissue inhibitors of MMP activity also exist and these include RECK (reversion-inducing, cysteine-rich protein with Kazal motifs), which encodes a membrane-anchored glycoprotein of about 110 kDa, with multiple EGF-like repeats and serine-protease inhibitor-like domains [14].

2. MMPs in pathology

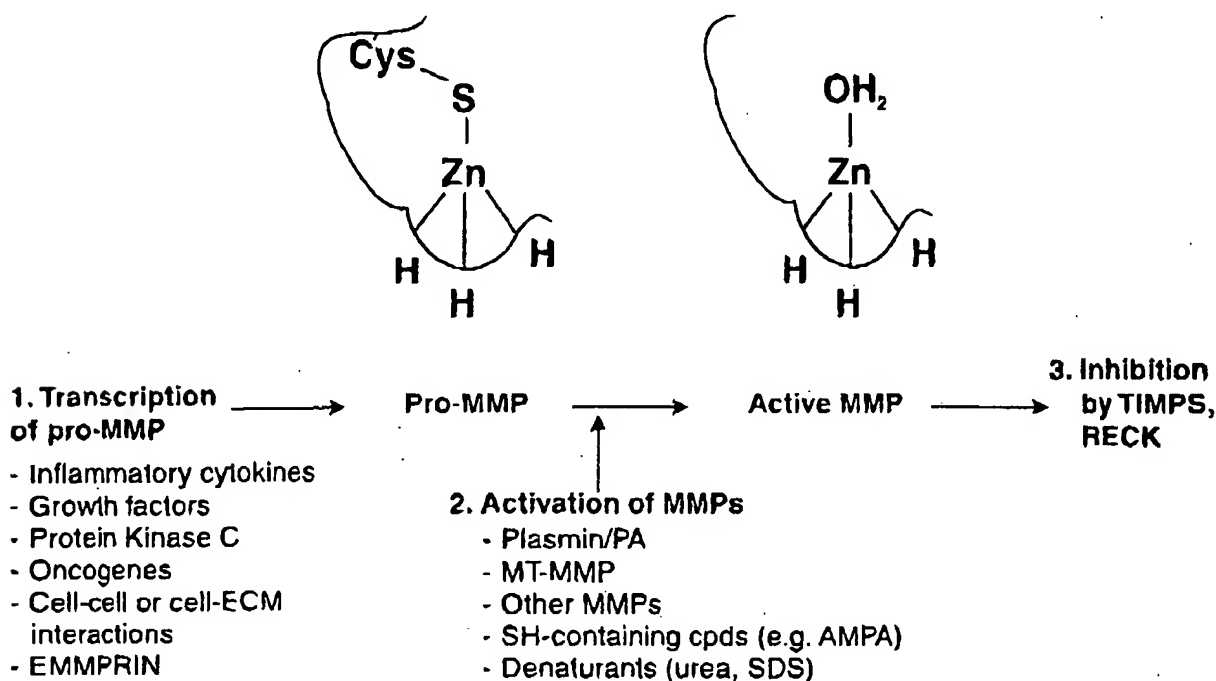
Despite the tight control of MMP activity, excess MMP production and the altered MMP:TIMP ratio is thought to be a key feature of several pathological processes

including rheumatoid arthritis, periodontal disease, fibrotic lung disease, liver cirrhosis, gastric ulcer, atherosclerosis, and aortic aneurysm (Table 1). MMPs are also implicated in leukocyte infiltration into tissues [15]. It is in the area of cancer metastasis, however, where the MMPs have gained the most prominence.

2.1 MMPs in cancer metastasis

Metastasis, the spread of cancer from a primary tumour to distant sites, must involve several changes in the cell behaviour: escape of cells from the primary tumour; intravasation (entry of cells into the lymphatic or blood circulation); survival and transport in the circulation; arrest in distant organs; extravasation and growth of cells to form secondary tumours in the new organ environment. Several such steps require MMPs [16], and a vast literature shows an association between metastasis and MMP expression. Of particular importance are MMP-2 and -9, because these gelatinases have broad specificity, in concert with a predilection for intact basement membrane Type IV collagen which surrounds blood vessels. Indeed, the expression of gelatinases is elevated in many types of human cancers including breast, colon, prostate and ovarian cancers [17]. Furthermore, the overexpression of MMPs in cell lines increases their

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Figure 2: Means by which the activity of MMPs is regulated, with details described in the text.

metastatic potential [18]. Conversely, the down-regulation of MMP expression, using approaches such as antisense ribozymes [19], or the overexpression of TIMPs [20,21], decreases the metastatic potential of several transformed cell types. In mice that are genetically deficient for MMP-2, implanted melanoma or Lewis lung carcinoma cells, are less tumorigenic [22,23].

Other MMPs are also implicated in cancer metastases and these include MMP-3 (stromelysin-1), MMP-7 (matrilysin) [24], MMP-10 (stromelysin-2) [25], and MMP-11 (stromelysin-3) [26].

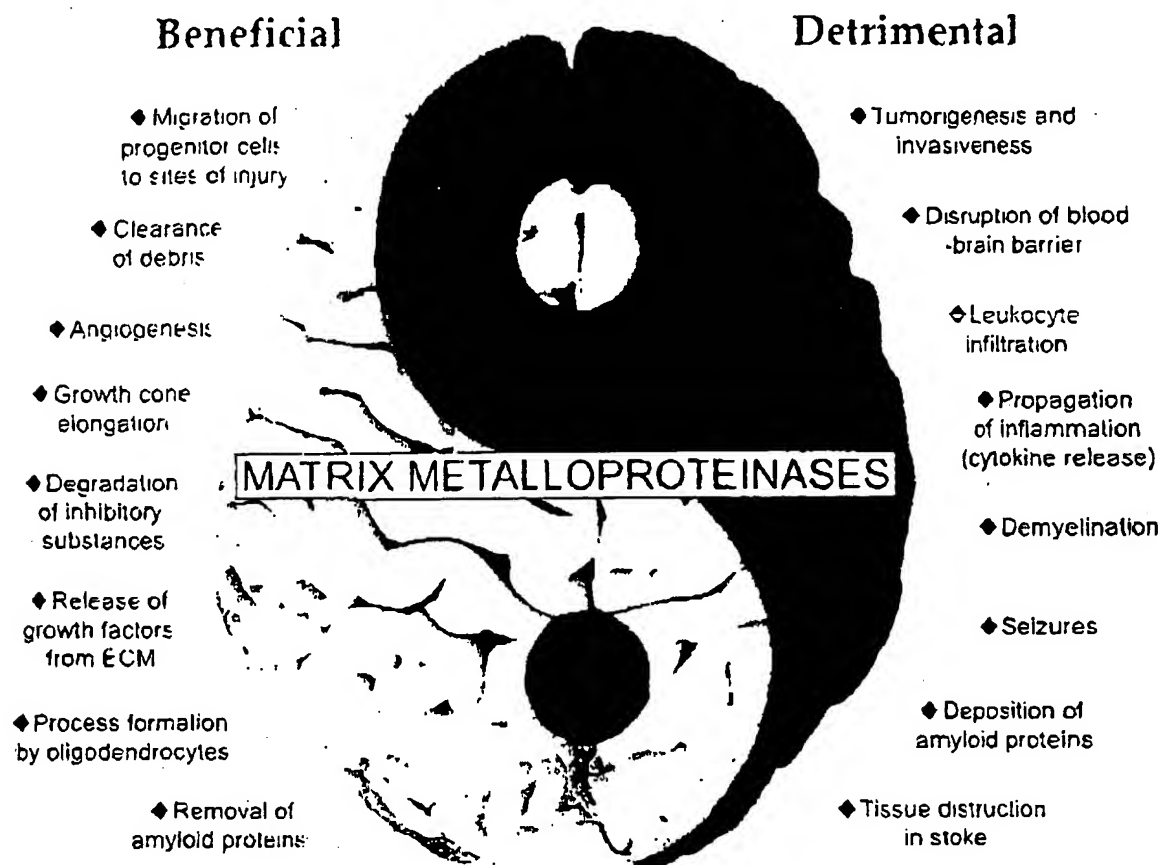
Somewhat unexpected is that in many tumours, MMPs are expressed not by tumour cells, but by stromal cells [27]. It appears that tumour cells induce the expression of MMP by stromal cells through a mechanism involving direct cell-cell contact and as a result, confines localised proteolysis at the tumour-stromal interface [28]. EMMPRIN (extracellular matrix metallo proteinase inducer), a transmembrane glycoprotein present on the surface of tumour cells, but not fibroblasts, and several other normal adult cells, is a potential tumour cell derived activator of stromal production of MMPs [29].

2.2 Trials of MMP inhibitors in cancer

Given the findings that MMPs regulate metastasis, a logical follow-up has been the use of MMP inhibitors in animal models of metastasis. Sledge *et al.* [30] reported that a broad-spectrum MMP inhibitor, batimastat (BB-94), significantly inhibited the local regrowth and metastasis of human breast cancer cells in a nude mouse xenograft model; this was also reported for many other tumour types including colon carcinoma cells [31]. Wang *et al.* [32] demonstrated that BB 94 reduced metastasis when fragments of human colon cancer were surgically implanted orthotopically on the colon of nude mice.

Human trials of MMP inhibitors in cancer are also in progress. In a Phase I study with BB-94 (batimastat) in patients with malignant ascites, half the patients did not re-accumulate ascites, or died up to 112 days after dosing [33]. Marimastat (BB-2516), an improved congener with oral efficacy, was administered to patients with advanced lung cancer to assess toxicity. The dose-limiting toxicity was polyarthrititis that persisted for up to eight weeks after drug administration [34]. A biological effect was found in a Phase II trial of marimastat in 415 patients with advanced ovarian, prostatic, pancreatic and colorectal cancers,

Figure 3: MMPs have both beneficial and harmful effects in the nervous system, a situation best illustrated by the 'yin and yang' of good and bad. All details can be found in the text.



and biological activity monitored by serial measurements of serum tumour markers [35].

3. Expression of MMPs in the CNS

Except during development, the expression of MMPs in the central nervous system (CNS) is generally low. The expression of MMPs is up-regulated in several pathological processes of the CNS and this has implicated MMPs in the pathogenesis of several CNS disorders [4]. The following sections review the findings that MMPs have a role in several CNS diseases.

3.1 MMPs in malignant gliomas

In common with non-CNS tumours described above, MMPs are implicated in the highly malignant CNS

tumour, malignant gliomas. Although gliomas rarely metastasise to the periphery, malignant gliomas are highly invasive locally within the CNS. The local invasiveness is a principal cause of mortality in the disease.

In vitro, cultured glioma cell lines express high MMP enzyme activity, and a good correlation has been found between the expression of MMP-2 and the degree of invasiveness *in vitro* [36, 37]; the production of MMP-2 in glioma cells is regulated by protein kinase C [37]. When the U251 human glioma line was stably transfected with the cDNA encoding MT1-MMP, MMP-2 activation was increased, leading to a concentration-dependent increased invasion across matrigel except when excessive MMP-2 activation was achieved [38]. The transfection of glioma cells with cDNAs encoding MMPs resulted in reduced invasive potential [39,40].

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In correspondence with cell culture studies, elevated levels of MMP-2 or MT1-MMP can also be detected in the homogenates of tissues resected from patients with gliomas. In particular, these levels increase with the grade of the disease [41-43]. Elevated levels of MMP-9 can also be detected in resected glioma specimens [44].

Recently, Lampert *et al.* [43] provided the most comprehensive study to date of the expression of MMPs in resected glioma specimens. By Northern blot analyses, normal human brain (i.e., non-neurological cases) had high levels of TIMP-2, while other TIMPs and MMPs were low. Compared with normal brain, the mRNA levels for MT1-MMP, MMP-2 and TIMP-1 were up-regulated progressively, with increasing grades of gliomas. Although a steady progression was not evident, the high grade glioblastoma multiforme also displayed increased levels of mRNA for MMP-9 and MT2-MMP. TIMP-2 and -3 did not vary with increasing grades. By immunohistochemistry, MT1-MMP and MMP-2 were found to be expressed by tumour cells, while MMP-9 was localised to both tumour and endothelial cells. This study provides the best evidence to date that MMP-2 and MT1-MMP are crucial in gliomas.

The tumour expression of MT1-MMP and MMP-2 in gliomas differs from the situation in the periphery, where many tumour types themselves do not produce MMPs, but rather induce the stroma to express these proteolytic enzymes (see Section 3). This may reflect either the poor ability of glioma cells to produce factors (e.g., EMMPRNs) that stimulate MMP expression, or may be due to an inability of non-transformed CNS cells to produce MMPs *in vivo* (although CNS-derived cells are good producers of MMPs *in vitro*). To resolve between these two possibilities, it may be instructive to measure MMP expression in brain parenchyma of patients where peripheral tumours (e.g., lung or breast) have metastasised into the CNS.

3.2 MMPs in multiple sclerosis and experimental allergic encephalomyelitis

At present, the most active research area of the consequence of aberrant MMP expression on CNS pathology is in the field of multiple sclerosis (MS). Over 20 years ago, it was determined that proteases capable of digesting myelin basic protein (MBP) were present in the cerebrospinal fluid of patients with MS [45,46] or in animals afflicted with experimental

allergic encephalomyelitis (EAE) [47], an autoimmune animal model of MS. More recently, some of these proteases were found to be MMPs. In this regard, MMP-2 has been found to be constitutively present in all CSFs while MMP-9 is found in the CSFs of patients with MS or other inflammatory neurological diseases, but not in control individuals [48-50]. With steroid treatment, MMP-9 levels in the CSF of MS patients were reduced [51].

3.2.1 MMPs are present in the brains of patients with MS and EAE

Immunohistochemically-identified MMPs are now found by several groups to be expressed in the autopsied brains of patients with MS. However, the cellular source of MMPs in the brains of MS is controversial. In the brains of subjects who died without any apparent neurological diseases, or in the apparently normal white matter of patients with MS, MMP-2 and -9 expression have been reported to be present predominantly in parenchymal microglia; some astrocytes were also positive [52,53]. In contrast, Cossins *et al.* [54] found that MMP-9 was rare, and could only be detected in some blood vessels. Anthony *et al.* [55] reported that MMP-9 was largely absent in the brain while MMP-2 antibodies stained neurones. MMP-3 and MMP-7 have been detected in microglia [52,54] while endothelial cells in the apparently normal human brain express MMP-3 and -9 [52].

In the MS lesions, microglia and astrocytes have both been reported to be positive for MMP-2, -3 and -9 [52-54], although the astrocyte expression has not been confirmed [55]. Noteworthy is the accord that immunohistochemically-identified MMP expression is predominantly in perivascular infiltrates [52-55].

Thus, further clarification is needed as to the CNS glia cell type that expresses MMPs in the normal state, and in the MS brain. It has to be noted that the technique of immunohistochemistry is relatively insensitive, and the absence of detectable immunohistochemical signal may be due to several factors including fixation conditions, the nature of the antibody itself, level of expression of the antigen, whether an amplification step is used during immunohistochemistry etc. Thus, a particular MMP member could well be present within neural cells but is below detection sensitivity. In common with MS, the development of EAE in rat was associated with a 3-fold increase in MMP activity in the CSF. Quantitative polymerase chain reaction

(PCR) of spinal cord revealed that of seven detectable MMP transcripts, matrilysin (MMP-7) was elevated 500-fold with onset of clinical symptoms, and peaked with maximum disease severity, while MMP-9 was elevated 5-fold. By immunohistochemistry, MMP-7 was localised to the invading macrophages within the inflammatory lesions of the spinal cord [56,57]. More recently, MMP-12 (metalloelastase) was also found to be elevated in inflammatory cells in EAE [58].

3.2.2 Leukocytes utilise MMPs to infiltrate the CNS parenchyma

The expression of MMPs, particularly MMP-7, -9 and -12, by perivascular leukocytes in MS and EAE, is thought to contribute to their infiltration into the CNS, since leukocytes are shown to depend on MMPs to penetrate barriers *in vitro* [59,60]. Also, macrophages from metalloelastase (MMP-12) knock-out mice were markedly diminished in their capacity to degrade ECM components; furthermore, these macrophages were essentially unable to penetrate reconstituted basement membranes *in vitro* and *in vivo* [61]. More recently, using an elegant *in vitro* model of the blood-brain barrier, lymphocytes treated with inhibitors of MMPs were found to be able to adhere to and diapedise between endothelial cells but were then unable to penetrate the next barrier consisting of an artificial basement membrane matrix [62].

3.2.3 Mechanisms by which MMPs contribute to disease production in MS

What can abnormally expressed MMPs do in the context of the MS disease process? Here, three effects are possible:

- blood-brain barrier (BBB) disruption
- degradation of myelin
- encephalogenicity

Rosenberg *et al.* [63] were the first to report that the injection of MMP-2 into rat brain increased capillary permeability, which was prevented by injection of TIMP-2. This has been confirmed [64]. In MS patients with enhancing lesions on MRI (an index of BBB dysfunction), CSF levels of MMP-9 were high, improved BBB permeability and decreased MMP-9 in the CSF both occurred with steroid treatment [51].

Elevated levels of MMPs can produce demyelination. In a delayed type hypersensitivity model of MS in the rat, the injection of highly purified activated MMP-7 and -9 into the brain parenchyma induced the loss of

myelin (MBP) staining [65]. The demyelination could be prevented by treatment of animals with an MMP inhibitor, BB-1101 [66].

Finally, the proteolysis of CNS proteins by MMPs may result in products that are harmful. Some of the MBP fragments of MMP-digestion are encephalogenic [67], several purified MMPs can degrade MBP, and these include MMP-2, stromelysin-1, interstitial collagenase, matrilysin, MMP-9 and MMP-12 [68,69].

3.2.4 Inhibitors of MMPs can ameliorate EAE

Given the accumulating evidence that MMPs have a role in the inflammation and tissue destruction in MS or EAE, it is not surprising that inhibitors of MMPs have been shown to ameliorate EAE. The first report indicated that GM6001, when administered daily to rats with EAE either from the time of disease induction or from the onset of clinical symptoms, suppressed the development or reversed clinical EAE [70]. Ro31 9790, given daily either at the time of disease induction or from 3 days post induction, reduced the clinical severity of adoptively transferred EAE. Administration of the inhibitor from the day of induction of active EAE prevented disease onset in 90% of animals. Histologically, decreased inflammation was seen [71]. BB-1101 was also effective in rat EAE, reducing the disease severity and weight loss [64].

Recently, an MMP inhibitor was found to block and reverse acute disease, and also to reduce the number of relapses and mean cumulative disease score in chronic relapsing animals. Demyelination and ghal scarring was also reduced, as was CNS gene expression of TNF- α and *fasl*. Expression of the beneficial cytokine, IL-4, was increased. In acute EAE, there was decreased inflammation, whereas this was not apparent in the chronic animals. Thus, the mechanisms of alleviation of disease in acute and chronic EAE may be different [72].

3.2.5 Interferon- β is an inhibitor of MMP-9 production

While clinical trials of MMP inhibitors in MS have yet to be initiated, the story of interferon- β (IFN- β) deserves to be mentioned. IFN- β has been shown to be efficacious in patients with MS, in that this drug reduces the number and severity of relapses, as well as the number of lesions that appear on MRIs. The mechanisms by which IFN- β is efficacious in MS is unclear (reviewed in [73]), but an interesting observation, published simultaneously by two groups, is that

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this drug reduces the production of MMP-9, and hence leukocyte trafficking *in vitro* [74,75]. Furthermore, MMP-9 production by T-lymphocytes increases in response to the chemokine MCP-1, and this was blocked by IFN- β [76]. Thus, although classic MMP inhibitors have not been formally subjected to clinical trials in MS, it is noteworthy that a clinically efficacious MS drug, IFN- β , has an important net result, at least *in vitro*, of decreasing MMP production and leukocyte trafficking.

3.2.6 The secretion of TNF- α is metalloproteinase-dependent

The pro-inflammatory cytokine, TNF- α , is first produced as a membrane-bound 26 kDa proform which is then proteolytically processed into the mature soluble 17 kDa form. Initially, this conversion was thought to be MMP-dependent [77,78], but more recent work has revealed that the TNF- α converting enzyme (TACE) is not an MMP, but a membrane-bound disintegrin metalloproteinase [79,80] belonging to the adamalysin metalloproteinases. These are zinc-dependent metalloproteinases containing both a disintegrin and metalloproteinase domains (ADAMs). TACE has been designated ADAM-17. Some MMPs have TACE-like activities. The purified catalytic domain of TACE is inhibited by peptide hydroxamate inhibitors and by TIMP-3, but not TIMP-1,2 or 4 [81]. The generation of mature TNF- α by MMPs, or by metalloproteinases such as ADAM-17, can lead to the propagation of a pro-inflammatory environment within the CNS.

3.2.7 Summary of MMPs MS

In summary, a great deal of evidence implicates the aberrant expression of MMPs in the pathology of MS. Although there is confusion as to the principal MMP member(s), or its cellular source(s) critical in MS, the larger picture would suggest that targeting MMPs is a reasonable approach to improve the treatment of patients with the disease.

4. MMPs in other disorders of the nervous system

Despite a lack of published research, MMPs are implicated in other CNS disorders.

4.1 MMPs in Alzheimer's disease

The 4 kDa amyloid protein, A β (or BA4), is the major constituent of amyloid plaques observed in the cortex

and hippocampus of the brains of patients with Alzheimer's disease (AD); its deposition is an early and constant event in the sequelae of the pathogenic cascade of the disease. The role of MMPs in A β deposition is controversial, in that MMPs have been reported to be either amyloidogenic [82] or to break down A β in the extracellular space, and thus prevent its accumulation. MMP-2 activity did not differ between AD and control hippocampal tissue but MMP-9 activity by zymography was increased 4-fold in AD [83]. Using immunohistochemistry, the presence of TIMP has been observed in the neuritic senile plaques and neurofibrillary tangles of the brains of patients with Alzheimer's disease [84]. These authors speculated that an MMP might be excessively produced or activated in selected locations of A β deposition and that, since TIMPs have high affinity for MMPs, the TIMPs localised to these sites.

More recently, it was suggested that the breakdown of APP to generate A β -containing C-terminal fragments was due to the action of non-MMP metalloproteinases, since its activity was not inhibitable by TIMPs [85].

In other research, Qiu *et al.* [86] studied how A β may be degraded by incubating the conditioned medium (CM) of metabolically-labelled A β -secreting cells with media of various cultured cell lines. Qiu *et al.* [86] found that the CM of a microglia line produced the most A β degrading activity. This was blocked by phenanthroline, but is likely not to be a MMP since the CM that was passed through a gelatin sepharose binding gel did not result in loss of A β -degrading activity. Similarly, another group [87] found that microglia, but not astrocytes, produced A β -degrading activity of about 200 kDa. The A β -degrading activity was blocked by batimastat and BB-2516, but not by TIMP, leading the authors to suggest that microglia may impair amyloid plaque formation by release of a non-MMP metalloproteinase that degrades soluble A β before polymerisation.

Presently, it remains unclear as to the role of MMPs, if any, in Alzheimer's disease. Clarification is needed as to whether MMPs are amyloidogenic or whether they prevent A β accumulation, and whether MMPs serve other functions in the disease.

4.2 MMPs in stroke

In experimental models of stroke, such as in middle cerebral artery occlusion (MCAO), an increase in MMP activity around the infarct zone has been detected: MMP-9 activity increased by 12h and MMP-2 by five

days [88]. Using subtractive cDNA library strategy for discovery of genes differentially expressed in focal stroke (MCAO), TIMP-1 mRNA was found to be robustly induced [89].

Elevation of MMP-9 was also detected in autopsy brain specimens by two day post infarction and remained elevated in cases dying months after the event. Increases in MMP-2 were subtle at two to five days, but again, were marked and significant in cases dying months after the event [90]. By immunohistochemistry, MMP-9 was strongly expressed by neutrophils in infarcted tissues from patients up to one week after an infarct (MMP-2 and -7 were less marked). From one week to five years, neutrophils were absent, and the large number of macrophages present were MMP-2 and 7 immunoreactive [55].

Recently, it was reported that the treatment in rats with a neutralising MMP-9 antibody 1h before MCAO reduced infarct size by 28% when compared to isotype control. Thus, MMP-9 is involved in the initial tissue destruction, and contributes to the rapidly developing brain injury that occurs after stroke [91].

The existing literature would suggest that MMPs are generally involved in certain aspects of stroke.

4.3. MMPs in other nervous system disorders

Aberrant expression of MMPs has been proposed to have a role in the pathogenesis of amyotrophic lateral sclerosis [92], seizures [93], bacterial [94] and viral meningitis [95] and in neuroblastomas [96,97]. Further studies are necessary to establish the mechanisms by which MMPs contribute, if at all, to these disorders.

5. The beneficial roles of MMPs in the nervous systems

A growing body of literature indicates that MMPs have important beneficial effects on the nervous system. During development, proteinases, including MMPs, are produced at the tips of growth cones to facilitate axonal elongation [98-100]. *In vitro*, MMP inhibitors attenuate growth cone activity, including the cessation of spike extension and ruffling [101]. Neurotrophic factors, including nerve growth factor increase the expression of MMP-2 and neurite outgrowth by DRG neurones [99] or induce the expression of stromelysin-1 in PC-12 cells [102].

MMPs may also modulate the migration of progenitor cells to their destinations during neural development.

At least *in vitro*, the migration of the oligodendrocyte progenitor, the O-2A cell, requires MMP activity [103]. The formation of oligodendroglial processes, a prerequisite event in myelin formation, is also dependent on MMP activity [104].

MMPs likely also play a role in the repair of the CNS during injury [4]. Besides the clearance of debris, the migration of precursor cells to the injury site to replenish lost cells could be MMP-dependent, as are the processes of angiogenesis, axonal regrowth or remyelination. MMPs may also be involved in the degradation of non-permissive ECM substrate (e.g., chondroitin sulfate proteoglycans) in order to allow facilitatory ECM substrates (e.g., laminins) to promote neurite extension; this was recently demonstrated elegantly in dorsal root ganglion neurones that were made to extend neurites on nerve segments [105].

In addition to the above, it is likely that MMPs also serve other useful functions in the nervous system, many of which remain to be elucidated. Any potential use of MMP inhibitors to treat neurological diseases will have to take into account the fact that MMPs also subservise useful functions in the nervous system.

6. The complexities of the use of MMP inhibitors to treat neurological diseases

It is obvious, as mentioned above, that the use of MMP inhibitors to treat neurological diseases will have side-effects. In the context of MS, for example, MMP inhibitors may be useful in attenuating inflammation or the destruction of myelin, but may retard attempts at remyelination. To facilitate ongoing recovery, the considered use of MMP inhibitors to treat MS for a brief period in order to curb acute inflammation, is perhaps desirable.

Another consideration is whether these agents get into the CNS. However, since the blood-brain barrier is leaky in many neurological diseases, penetration into the CNS is likely not to be a problem, especially as many MMP inhibitors are small molecules and are highly lipophilic.

A major issue that needs to be resolved is the specificity of the MMP inhibitors. Although labelled as 'MMP inhibitors', it has become evident that many of the hydroxamate based inhibitors of MMPs also affect the larger family of metzincin metalloproteinases [106]. These are Zn-dependent metalloproteinases where the active site zinc is co-ordinated by three

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histidine residues in a conserved orientation: HXXHXXGXXH. There is also a distinct β -turn delineated by a methionine residue which is important in maintaining the conformation of the active site. Metzincin subgroups include the MMPs, astacins (e.g., bone morphometric protein), serpins, and adamalysins. The last are subgrouped into ADAMs (a disintegrin and metalloproteinase) and snake venom-like metalloproteinases (reviewed in [107]). ADAM-17 (TACE) has been described above, while ADAM-10 (Kuzhanlian), has an important role in notch signalling and CNS development [108,109]. Thus, the issue of whether the 'MMP inhibitors' target the larger family of metzincin metalloproteinases needs to be considered, since this obviously will extend the range of possible side-effects.

7. Conclusion

MMPs play a role in neurological diseases; evidence is good for their involvement in malignant gliomas and multiple sclerosis. Targeting MMPs is likely to produce several therapeutic benefits, however, this has to be balanced against the observation that MMPs have many beneficial actions in the CNS. Current 'MMP inhibitors', with the exception of TIMPs, are likely to target members of the larger metzincin metalloproteinase family and their association with several side-effects is probable. Finally, it would be important to develop selective inhibitors not only against the MMP family in contrast to all metzincin metalloproteinases, but also against individual MMP family members, so that specific inhibition of a particular MMP can be achieved. In so doing, the incidence of side-effects can be reduced in tandem with increased drug potency.

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sequences identified, it might be possible, using either conventional pronuclear injection techniques or viral vector technology, to create transgenic montane voles that carry a functional OT or V_{1a} receptor transgene with expression driven by prairie vole promoters. This might result in montane voles in which the pattern of neuropeptide-receptor gene expression and potentially, social behavior have been altered. If successful, and provided that the appropriate transcription factors and second-messenger pathways are in place, these experiments should demonstrate the behavioral consequences of altered receptor expression and potentially establish a link between specific genes and monogamy in rodents.

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Matrix metalloproteinases and diseases of the CNS

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Matrix metalloproteinases (MMPs) are increasingly being implicated in the pathogenesis of several CNS diseases. In multiple sclerosis, MMPs could be responsible for the influx of inflammatory mononuclear cells into the CNS, contribute to myelin destruction and disrupt the integrity of the blood–brain barrier; in Alzheimer's disease, MMPs might mediate the deposition of amyloid β -proteins; and MMPs are known to contribute to the invasiveness of malignant glioma cells and might regulate their angiogenic capacity. Nonetheless, MMPs could also have beneficial roles in recovery from CNS injury. Therefore, both the identity of the MMP and its cellular origin could determine whether disease pathogenesis or regeneration occurs, and thus synthetic MMP inhibitors might be valuable for treating some CNS diseases.

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MATRIX METALLOPROTEINASES (MMPs) are proteolytic enzymes that are involved in the remodelling of the extracellular matrix (ECM) in a variety of physiological and pathological processes. The MMP family consists of at least 18 members (Table 1) that

have common propeptide and N-terminus catalytic domains (Fig. 1). Additional, fibronectin-like repeats, transmembrane domains and C-terminus hemopexin-like domains allow categorization of MMPs into the collagenase, gelatinase, stromelysin and membrane-type

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TABLE 1. Members of the matrix metalloproteinase (MMP) family

Group	Members	MMP number	Main substrates
Collagenases	Interstitial collagenase	MMP-1	Fibrillar collagens
	Neutrophil collagenase	MMP-8	Fibrillar collagens
	Collagenase-3	MMP-13	Fibrillar collagens
	Collagenase-4	MMP-?	Not known
Gelatinases	Gelatinase A	MMP-2	Gelatin, Types IV, V collagens, fibronectin
	Gelatinase B	MMP-9	Gelatin, Types IV, V collagens, fibronectin
Stromelysins	Stromelysin-1	MMP-3	Laminin, non-fibrillar collagens, fibronectin
	Stromelysin-2	MMP-10	Laminin, non-fibrillar collagens, fibronectin
	Matrilysin	MMP-7	Laminin, non-fibrillar collagens, fibronectin
	Stromelysin-3	MMP-11	α 1 proteinase inhibitor (serpin)
MT-MMPs	MT1-MMP	MMP-14	Pro-MMP-2, collagens, gelatin
	MT2-MMP	MMP-15	Pro-MMP-2, collagens, gelatin
	MT3-MMP	MMP-16	Pro-MMP-2, collagens, gelatin
	MT4-MMP	MMP-17	Pro-MMP-2, collagens, gelatin
Others	Matloelastase	MMP-12	Elastin
	Enamelysin	MMP-?	Not known
	Xenopus MMP	MMP-?	Not known
	Not known	MMP-19	Aggrecan

Abbreviations: MMP, matrix metalloproteinases; MT-MMPs, membrane-type matrix metalloproteinases. There is no MMP-4, -5 or -6. MMP-? refers to members where the numerical designation has not been assigned.

MMP (MT-MMP) subfamilies. Physiologically, MMPs are thought to be important in wound healing, ovulation, blastocyst implantation, bone growth and angiogenesis. In contrast, MMPs appear to have a pathological role in a variety of disease processes such as tumor invasion and metastasis, rheumatoid arthritis,

periodontal disease and atherosclerosis. This review highlights the evidence that MMPs also have a pathogenic role in several CNS diseases, paying particular attention to multiple sclerosis, Alzheimer's disease and malignant gliomas. The possible role of MMPs in facilitating CNS recovery is also discussed.

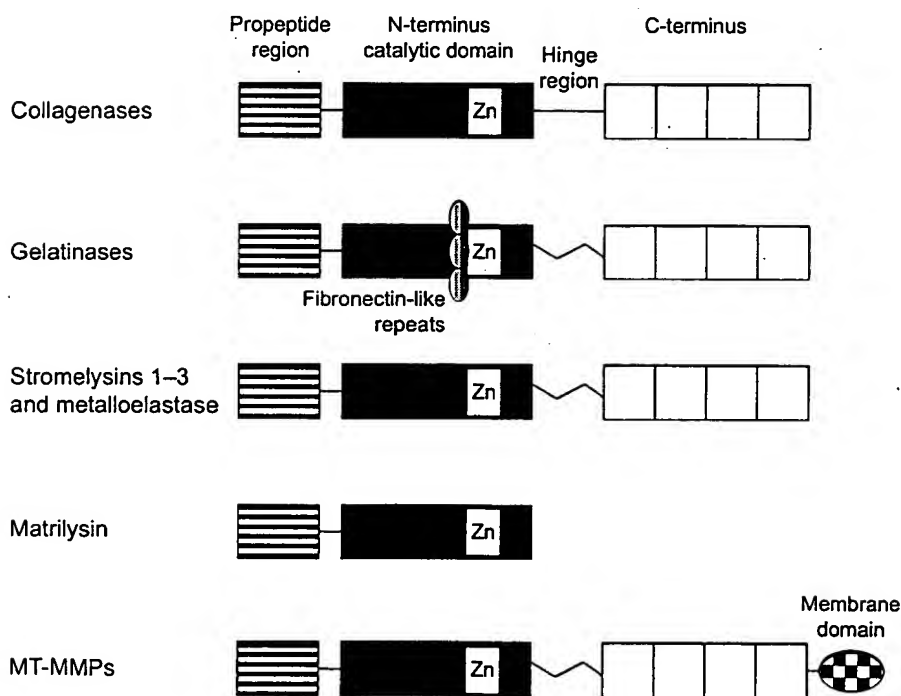


Fig. 1. Modular domain structures of the matrix metalloproteinases (MMPs). The N-terminus propeptide region has about 80 amino acids and is common to all MMPs. An invariant cysteine residue in the propeptide domain ligates to the zinc ion (Zn) of the catalytic domain and blocks its activity. The C-terminus domain, present in all MMPs except matrilysin, has a high level of homology with members of the hemopexin family. C- and N-terminus domains are connected by a linker region or hinge that is short in collagenases and long in other MMPs².

Regulation of MMP activity

Because MMPs can catalyze the degradation of all the protein constituents of the ECM, it is important that their activities are kept under tight control to prevent tissue destruction. The activity of MMPs is regulated in three ways: gene transcription, proenzyme activation and by the action of tissue inhibitors of metalloproteinases (TIMPs).

Most MMPs are not constitutively expressed, but gene transcription can be induced by stimuli including phorbol esters, growth factors, inflammatory cytokines, oncogene products and cell-ECM or cell-cell interactions. In most cases, these stimuli induce the expression of members of the *c-fos* and *c-jun* proto-oncogene families, whose products form the homo- and heterodimeric forms of the activator protein-1 (AP-1) transcription factors. The AP-1 factors bind to specific DNA sequences (called AP-1 binding sites, phorbol ester response elements or TREs) in gene promoters or enhancers, thereby affecting transcription. The promoter regions of MMP-9, stromelysins and collagenases contain the AP-1

sequence, whereas MMP-2 does not, suggesting that it could be constitutively expressed.

The MMPs are initially expressed as inactive pro-MMP zymogens where a zinc atom present in the catalytic domain is bound to a cysteine residue in the pro-peptide region. Activating factors disrupt the cysteine-zinc interaction ('cysteine switch') and thus expose the catalytic site; the result is a partially active intermediate form of the enzyme that can cleave the propeptide region by autocatalysis and render the enzyme fully active¹. An important physiological activator of pro-MMPs is plasmin, a serine proteinase that is generated from plasminogen by the action of tissue- or urokinase-plasminogen activator (uPA). Activation of the MT-MMPs also requires removal of a propeptide, but this is catalyzed by a serine proteinase, furin. Activation pathways can co-operate, leading to the activation of additional downstream MMPs such as MMP-9, as shown in Fig. 2. The activation apparatus is localized on the surface of the cell, and an important consequence of this is that proteolysis is greatest in the immediate pericellular environment, where it can influence cell-cell and cell-ECM interactions.

Following activation, MMPs can be regulated by the formation of tight, 1:1 non-covalent complexes with TIMPs (Ref. 2). The four known TIMPs share many properties but also have distinct activities (Table 2), suggesting that they might have specific physiological roles. The functional relationship and interaction between TIMPs and MMPs is complicated: TIMPs form complexes with the inactive pro-enzyme forms of gelatinases via interactions that are different from those between TIMP and the active form of these enzymes. These complexes, such as the pro-MMP-9/TIMP-1, pro-MMP-2/TIMP-2 and pro-MMP-2/TIMP-3 complexes, control the rate at which physiological factors activate MMPs. For example, TIMP-2, when complexed with MMP-2, has been shown to be an adaptor molecule that allows pro-MMP-2 to become associated with MT-MMPs (Ref. 2). Thus, an important aspect of TIMP-2 (and TIMP-3) function is that cell behavior critically depends on the stoichiometry between TIMP-2, MT-MMP and MMP-2. Low concentrations of TIMP-2 favor pro-MMP-2 activation, and high concentrations block activation by neutralization of all available MT-MMP binding sites.

The TIMP genes are also regulated: because the promoter region for TIMP-1, but not TIMP-2, contains an AP-1 site, the MMPs and their inhibitors can be coordinately induced. However, in some cases opposite

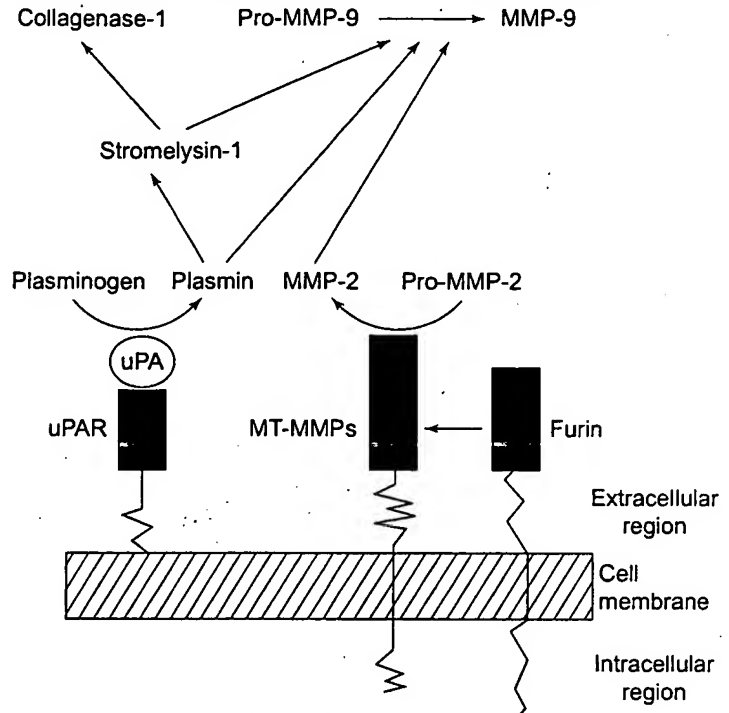


Fig. 2. Cascade of matrix metalloproteinase (MMP) activation at the cell surface. The co-ordinate activation of several MMPs is initiated by the formation of plasmin. Plasmin is produced from plasminogen by the action of urokinase plasminogen activator (uPA) that is anchored by its receptor, uPAR. Plasmin can activate MMP-9 and stromelysin-1, and the latter can in turn activate other MMPs, including MMP-9 and collagenase-1, thus amplifying and broadening the activation cascade. MMP-2 is activated by membrane-type MMPs (MT-MMPs) that are activated by furin proteinases.

patterns of regulation of MMPs and TIMPs have been described. For example, in fibroblasts and endothelial cells, TGF- β upregulates TIMP-1 expression and represses stromelysin and collagenase expression⁴. Additionally, in human peripheral blood monocytes, interleukin (IL)-10 enhances TIMP-1 production but decreases MMP-9 biosynthesis; this was cell-type specific, because IL-10 did not affect TIMP or MMPs in fibroblasts⁵.

Under physiological conditions, MMP activity is precisely controlled; however, excess MMP production and activation is thought to be a key feature of the pathology of many inflammatory and malignant diseases.

TABLE 2. Properties of tissue inhibitors of metalloproteinases (TIMPs)*

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Chromosome gene location (human)	Xp11.23-11.4	17q23-2.5	22q12.1-13.2	?
Protein (kDa)	28	21	24	22
RNA (kb) [†]	0.9	3.5(1.0)	4.5(2.8,2.4)	1.2
Major sites	Ovary, bone	Lung, brain	Kidney, decidua, brain	Brain, heart
Expression	Inducible	Largely constitutive	Inducible	?
Predominant form of expressed molecule	Secreted	Secreted	ECM-associated	Secreted
Pro-MMP complex	MMP-9	MMP-2	MMP-2	?
Inhibition of MT-MMP	No	Yes	Yes	?
Inhibition of gelatinases	Yes	Yes	Yes	Yes

*Reviewed in Refs 2,3. †, numbers in brackets denote possible splice variants. Abbreviations: ECM, extracellular matrix; MMP, matrix metalloproteinases; MT-MMP, membrane-type matrix metalloproteinases; ?, unknown.

MMPs in multiple sclerosis

Several lines of evidence suggest that MMPs are important in the pathogenesis of multiple sclerosis (MS). The demonstration that proteases capable of catalyzing the degradation of myelin are present in the cerebrospinal fluid (CSF) of patients with MS or its animal counterpart, experimental allergic encephalomyelitis (EAE) (Ref. 6), is long standing; some of these have been identified as MMPs (Ref. 7). Furthermore, various members of the MMP family can be found in the brains of patients with MS (Refs 8,9), or animals with EAE (Ref. 10). All cell types of the CNS are potential sources of MMPs. *In vitro*, neurons¹¹, astrocytes^{11,12}, microglia^{11,13} and oligodendrocytes¹⁴ express various MMP family members, and the production of MMPs by neural cells can be up-regulated by several inflammatory cytokines. In autopsied brain specimens of normal subjects, MMP-like immunoreactivity (for MMP-1, -2, -3 and -9) is localized in microglia and astrocytes. MMP expression is up-regulated in these cells, and also in perivascular macrophages that are present in active brain lesions of patients with MS (Refs 8,9). In the normal rat, transcripts encoding at least seven MMPs are found in spinal cord extracts: these are MMP-2, -3, -7, -9, -11, -13 and -14. In animals afflicted with EAE, MMP-9 showed a modest increase, whereas MMP-7 (matrilysin) was elevated >500-fold¹⁰. By immunohistochemistry, matrilysin was found to be up-regulated in perivascular macrophages and astrocytes of rats with EAE (Ref. 10).

Concurrent with the early evidence of proteases in the CSF of patients with MS or animals with EAE, Brosnan *et al.*¹⁵ reported that several non-specific protease inhibitors reduced the symptoms of EAE in Lewis rats. More recently, attempts to alleviate MS and EAE have focused on the use of synthetic inhibitors of MMPs. A hydroxamate inhibitor of MMP, GM6001, was found to suppress the development of, or reverse established EAE in rats when administered either from the time of disease induction or from the onset of clinical symptoms, respectively¹⁶. Another hydroxamate inhibitor of MMP, Ro31-9790, reduced the clinical severity of adoptively transferred EAE, and prevented the onset of the disease in 90% of animals¹⁷. A broad-spectrum MMP inhibitor, BB-1101, also reduced weight loss and severity of EAE (Ref. 18).

The mechanism of action of MMP inhibitors in EAE could include the inhibition of MMPs that have been secreted by inflammatory cells or the reduction of tumor necrosis factor- α (TNF- α) production. T cells produce several MMPs, and the migration of T cells and macrophages across ECM barriers requires their production of MMP-9 (Refs 19,20). Thus in MS or EAE, MMP inhibitors might act by preventing the influx of inflammatory cells across the basement membrane or ECM barrier that surrounds cerebral endothelium. Indeed, it has been shown that the inhibition of MMP-9 activity of T lymphocytes is a major mechanism of action of interferon-beta, a drug used clinically in MS (Refs 19,21).

The blocking of the conversion of a 26 kDa membrane-anchored protein, pro-TNF- α , to the mature 17 kDa secreted protein is another mechanism by which MMP inhibitors might influence MS or EAE. In addition to being pro-inflammatory, TNF- α can damage oligodendrocytes and myelin both *in vitro* and *in vivo*. However, recent work suggests that the major TNF- α -

converting enzyme is not an MMP: the conversion is not inhibited by TIMPs, and none of the purified MMPs tested cleaved pro-TNF- α with appropriate specificity. Indeed, the TNF- α -converting enzyme was recently purified and cloned by two independent groups, and was identified as a membrane-bound disintegrin metalloproteinase of the adamalysin family^{22,23}.

Yet another deleterious action of MMPs in MS and EAE relates to their effects on the integrity of the blood-brain barrier (BBB): the injection of MMPs into the rat brain increases capillary permeability and can be prevented by TIMP-2 (Ref. 24); others have found that an intracerebral injection of MMP leads to extensive leakage of the BBB (Refs 18,25). In particular, Matyszak and Perry²⁵ demonstrated that the breakdown of the BBB, and the recruitment of T cells into the lesion site caused by a delayed-type hypersensitivity response, was reduced by an MMP inhibitor. The authors also found that the myelin loss caused by the delayed-type hypersensitivity response was significantly attenuated by the MMP inhibitor.

In summary, the aberrant expression of MMPs might be important in neuro-inflammatory diseases such as MS, and thus MMP inhibitors might enable advancements to be made in the therapeutic treatment of these diseases.

MMPs in Alzheimer's disease

An early feature of Alzheimer's disease (AD) is the neocortical deposition of amyloid- β proteins ($A\beta$), which arise from the proteolytic cleavage of a large integral membrane protein, β -amyloid precursor protein (APP). Extensive studies of APP metabolism have shown that it can be cleaved by the action of multiple proteases, called secretases, that remain to be characterized. The protein APP is cleaved predominantly by an α -secretase, and yields a large N-terminus fragment that is secreted (termed sAPP α or α -APPs) and a membrane-associated 10 kDa C-terminus derivative; this mode of cleavage is not thought to be amyloidogenic, because the cleavage occurs within the $A\beta$ region. An alternative pathway utilizes β -secretase, leading to the secretion of β -APPs, and the retention of a 12 kDa membrane-bound fragment that is thought to give rise to $A\beta$ following cleavage by γ -secretase. In addition to the α - and β -secretory pathways, a substantial portion of full-length APP appears to be degraded in the lysosomal system²⁶.

When mixed glial and neuronal hippocampal cultures were exposed to $A\beta$, an increase in the expression of several MMPs was found²⁷, suggesting that MMPs might be increased in AD-afflicted brain tissue. An early report that a calcium-dependent metalloproteinase, reminiscent of gelatinases, is expressed at higher levels in hippocampus from AD patients compared to controls is consistent with this idea²⁸. Furthermore, it has been reported by Miyazaki *et al.*²⁹ that MMP-2 can cleave $A\beta$ at the α -secretase site, and conversely that APP has a domain that inhibits MMP-2 activity. Independently, it has been shown that MMP-2 can cleave the full-length APP (Ref. 30). These results suggest that MMP-2 might produce α -APPs at the plasma membrane or degrade $A\beta$ in the ECM, or both. In either case, $A\beta$ accumulation would be prevented. However, others³¹ were unable to reproduce these results and reported that MMP-2 could

have β -secretase activity, and thus would be amyloidogenic. Other candidate α - and β -secretases have been proposed based on their ability to cleave substrates that mimic the native APP cleavage sites³². Whether or not MMP-2 can cleave APP *in vivo* is still under debate^{29,33}.

Other corroborating evidence is that TIMPs have been found in the amyloid plaques and neurofibrillary tangles of AD-afflicted brain tissue³⁴. These authors speculated that an MMP might be excessively produced or activated in selected locations of A β deposition, and that TIMPs might become localized in these sites because of their high affinity for MMPs. It is also conceivable that the deregulation of TIMPs contributes to A β production in AD.

The role of MMPs and TIMPs in AD remains to be fully determined. It is important to clarify whether or not MMPs (or TIMPs) are amyloidogenic or prevent A β accumulation, because they are candidates for therapeutic or preventative modulation.

MMPs in malignant gliomas

The classical area of MMP research is tumor invasion and the metastasis of systemic cancers. Invasion requires at least two changes in cell behavior: first, the affinity of cells, either for each other or for the ECM, must decrease in order to allow the release of cells from the primary tumor; second, the surrounding ECM must be remodeled by the local production of proteolytic enzymes in order to allow for cellular migration. Various MMPs are elevated in many types of human cancers including breast, colon, prostate, bladder, ovarian and brain neoplasms (reviewed in Ref. 35), and several synthetic inhibitors of MMPs have been shown to reduce tumor invasion and metastasis in animal models. Furthermore, the overexpression of MMPs (including MT-MMPs) in cells increases their metastatic activity, whereas the overexpression of several TIMPs reduces their invasiveness³⁶. Surprisingly, the increased MMP expression in most tumors derives from host stromal cells rather than the tumor cells themselves, highlighting the importance of tumor-stromal interactions³⁷.

Malignant gliomas are the most common type of malignant brain tumors in adults, and are second only to stroke as the leading cause of death from neurological disease. Although malignant gliomas rarely metastasize outside of the CNS, they are extremely invasive tumors. Local dissemination inevitably occurs and is a major cause of the high morbidity and mortality of the disease; MMPs might be responsible for this highly invasive behavior. A strong correlation can be found between the invasiveness of glioma cells *in vitro* or *in vivo*, and their production of MMP-2, MMP-9 or MT-MMP (Refs 38-40). It has been shown that the expression of MMP-2 by glioma cells correlates with their invasive rate⁴⁰ and, in a few patients, accounts for metastasis outside the CNS (Ref. 41).

In contrast to MMPs, TIMP-1 and -2 concentrations can be low in malignant gliomas⁴², suggesting that decreased inhibition of MMP could contribute to their dysregulation in these cells; whether or not all four forms of TIMPs are altered in gliomas remains to be determined. Furthermore, because TIMPs can be mitogenic⁴³, the potential effects of MMPs (or TIMPs) on glioma proliferation might be important. Finally, excessive MMP activity might also increase the

angiogenic capacity of those tumors that are highly vascularized.

Other non-MMP proteinases might be important in glioma biology (reviewed in Ref. 44). The concentration of uPA correlates with the grade of malignant gliomas, as do the elevated concentrations of cysteine proteases such as cathepsin B. Whether these proteinases have direct or indirect actions on proliferation, invasion or angiogenesis is unclear, because they can work upstream in a cascade that converts MMP zymogens to active proteases (Fig. 2).

The expression of MMPs is upregulated in many malignant gliomas, and this could contribute to the highly invasive phenotype of glioma cells. Inhibitors of MMPs might have efficacy in patients with malignant gliomas, and are currently being tested in several clinical trials. Whether these will be effective alone or require combination therapy with cytotoxic drugs or radiotherapy remains to be determined.

MMPs in other neurological diseases

MMPs can cause an increase in capillary permeability, and have been shown to produce brain edema that is secondary to ischemic and hemorrhagic brain injury. In rats, treatment with an MMP inhibitor reduced brain edema following intracerebral hemorrhage⁴⁵, suggesting that interference in some of the functions of MMPs might improve recovery from stroke. MMPs could also contribute to the pathogenesis of amyotrophic lateral sclerosis⁴⁶, although this needs to be established. It is likely that the number of neurological diseases in which MMPs are important will increase dramatically.

Is there a role for MMPs in facilitating CNS recovery?

In addition to the diseases discussed, MMPs are upregulated following other types of insult to the nervous system, such as penetrating injury or peripheral nerve transections⁴⁷. The up-regulation of MMPs following almost all CNS injury raises the possibility that some MMPs could function to enhance the recovery of the CNS. What might some of these functions be? It is likely that following an injury that leads to cell death, some degradation of the ECM must occur in order for the environment to be remodeled, and MMPs could fulfil this role. Although the adult CNS does not contain a well-defined parenchymal ECM, an ECM barrier does exist in the basement membrane that surrounds cerebral blood vessels, and a diffuse and amorphous mixture of ECM molecules can be found throughout the CNS (Ref. 48). New blood vessels are likely to be required to facilitate recovery from various CNS insults, and MMPs might play a role in angiogenesis. When mononuclear phagocytes are recruited to the lesion, these might utilize MMPs to facilitate their migration or to engulf debris, or both. With respect to cell migration, neural-cell precursors might require MMPs in order to migrate to the lesioned area and replenish lost cells; a precursor cell for oligodendrocytes has been demonstrated to require MMPs for motility⁴⁹. New axonal growth and synaptic reconstructions need to be established and their extension through the brain matrix might require MMPs; indeed, stromelysin-1 has been found to mediate the motility of growth cones through basal laminae⁵⁰. It has been found that oligodendrocytes utilize MMP-9

Box 1. Potential consequences of matrix metalloproteinase (MMP) expression in the mature nervous system

Undesirable effects

- Breakdown of the blood-brain barrier
- Demyelination
- Cytokine production and propagation of an inflammatory response
- Deposition of amyloid proteins
- Tumor invasion, metastasis and angiogenesis
- Inappropriate degradation of extracellular matrix leading to alteration of structural integrity

Beneficial actions

- Clearance of debris following injury
- Remodeling of ECM for cell migration and axonal elongation
- Release of growth factors anchored on the ECM
- Breakdown of amyloid proteins
- Angiogenesis
- Process formation by oligodendrocytes

to extend their processes¹⁴, which is a prerequisite for developmental myelin formation and remyelination. The upregulation of MMPs in the MS brain or in other CNS pathologies, might not always be harmful, and thus it is important to be able to discriminate between the beneficial and deleterious effects of MMPs. Lastly, the remodeling of the ECM might release several neurotrophic factors, such as basic fibroblast growth factor, that are anchored on the ECM. Thus, there are several mechanisms by which the production of MMPs, following insults to the nervous system, might be beneficial. These possibilities, and the role of MMPs in the disease described earlier, are summarized in Box 1.

Conclusions

The presence of MMPs in the CNS is well documented. Their functions during neural development and in neurological diseases are beginning to be elucidated, and it is evident that MMPs have significant effects on the brain micro-environment. The issue of MMPs as causative factors in disease is an active area of investigation, but their possible role as facilitators of CNS recovery needs greater consideration. Because the activation of MMP cascades is primarily conducted at the cell surface, proteolysis is likely to be focally restricted to the pericellular environment. This means that although the MMPs have overlapping substrate preferences, the nature of the particular MMPs present, and the identity and localization of the cells that are expressing MMPs, might critically determine whether disease or recovery is favored. For example, it is conceivable that matrilysin produced by invading macrophages contributes to debris clearance or demyelination in MS, whereas MMP-9 that has been elaborated by oligodendrocytes represents an attempt by these cells to regenerate their processes and remyelinate neurons. The potential use of MMP inhibitors to treat CNS diseases is an exciting prospect, but it is important to balance the risk with the benefits of this treatment. The MMP-deficient mice that are

being generated in several labs will be extremely valuable in determining the precise roles of individual MMPs in CNS disease and repair.

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CORRIGENDUM

In the Review article entitled 'Reactive astrocytes: cellular and molecular cues to biological function', by J.L. Ridet, S.K. Malhotra, A. Privat and F.H. Gage, which was published in the December 1997 issue of *TINS*, an element in Fig 1B was wrongly labelled. 'Li' should be 'LI'.

We apologize to the authors and readers.

Possible Involvement of a Tumor Necrosis Factor (TNF)-like Mediator as an Endogenous Pyrogen in Fever Induction by *Nocardia rubra* Cell Wall Skeleton (N-CWS)

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Tumor necrosis factor (TNF), a cytokine produced in macrophages, also acts as an endogenous pyrogen (EP). To investigate whether TNF has a role in the fever induced by *Nocardia rubra* cell wall skeleton (N-CWS), the relationship between fever and TNF production was studied in guinea pigs.

N-CWS injected i.v. to guinea pigs caused biphasic fever and had L-929 cell-killing activity which resembled that of TNF in the sera 30 min before the first phase of fever appeared. *In vitro*, L-929 cell-killing activity was demonstrated in the culture supernatant of guinea pig peritoneal macrophages pretreated with N-CWS, and the activity increased dependently on N-CWS concentration or culture duration. When the supernatant of the macrophages was fractionated by gel filtration and each fraction was assayed for fever-inducing and L-929 cell-killing activities, the fraction with the cell-killing activity also induced fever with characteristics similar to that by i.v. injection of N-CWS in guinea pigs.

These results suggest that TNF acts as an EP on the fever induced by N-CWS in guinea pigs.

Keywords N-CWS (*Nocardia rubra* cell wall skeleton); fever; TNF (tumor necrosis factor); endogenous pyrogen

N-CWS is the cell wall skeleton prepared from a gram-positive bacterium, *Nocardia rubra*. This substance has a potent anti-tumor activity in several experimental animal models^{1,2)} and in humans.³⁻⁶⁾ Fever was a side effect of N-CWS in clinical trials, and also in such experimental animals as rabbits and rats.

In this connection, it has been shown that endogenous pyrogen (EP) produced in phagocytes stimulated with microorganisms induces fever by enhancing the production of prostaglandins (PGs) in the hypothalamus or adjacent regions.⁷⁻⁹⁾ It has also been reported that EP has many other biological activities,¹⁰⁻¹⁶⁾ and that their profiles are similar to those of interleukin-1 (IL-1). Because of this, EP is accepted to be IL-1. Recently, Dinarello *et al.*¹⁷⁾ reported that tumor necrosis factor (TNF) had EP activity in an experiment in which fever was induced by i.v. injection of recombinant human TNF (r-TNF α) in rabbits. Furthermore, they used r-TNF α to induce IL-1 production *in vitro* in human monocytes, and accordingly, concluded that two EPs were involved in the fever induced by TNF; one, TNF itself, and the other IL-1 produced by TNF. This indicates the importance of TNF as an EP in the fever producing process.

In our previous study, i.v. N-CWS caused biphasic fever in rats, rabbits, and guinea pigs, and the fever resembled that induced by r-TNF in rabbits in the study by Dinarello *et al.*

The present study was performed to investigate whether TNF acted as an EP in the fever induced by N-CWS.

Experimental

Animals Six Hartley male guinea pigs weighing 350–700 g were purchased from Shizuoka Agricultural Cooperative Association. The animals were housed under room temperature of $20 \pm 5^\circ\text{C}$; relative humidity, $60 \pm 5\%$; and lighting controlled to give 12 h of light and 12 h of darkness.

Chemicals Cell wall skeleton of *Nocardia rubra* (N-CWS) was prepared¹⁸⁾ by Fujisawa Pharmaceutical Co., Ltd. and suspended in physiological saline. Lipopolysaccharide (LPS) contamination in N-CWS was assayed by using the Limulus amoebocyte lysate assay and was found to be negligible, i.e. 0.36 ng per mg of N-CWS. Human γ -globulin (h- γ G; Sigma), bovine serum albumin (BSA; Sigma) and cytochrome c (cyt-c; Sigma) were used as molecular mass markers. Hank's balanced salt solution (HBSS; Nissui Pharmaceutical) was used as the culture medium.

Measurement of Rectal Temperature Guinea pigs were lightly restrained by hand, and a thermister-probe (TF-DN type; Termo Co., Ltd.) was inserted about 50 mm into the rectum. The rectal temperature was measured for 1 min at intervals of 30 min.

Blood Sampling Blood was taken by heart puncture 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 or 3.0 h after an i.v. injection of N-CWS into guinea pigs, and sera was separated by centrifugation for assay of L-929 cell-killing activity.

Isolation and Culture of Macrophages Guinea pig macrophages were isolated by a modification of the method of Ohishi *et al.*¹⁹⁾ Liquid paraffin was injected i.p. into guinea pigs to release cells into the peritoneal fluid, and 3 or 4 d later, the cells were collected, suspended in HBSS without serum, and incubated at 37°C in a humidified atmosphere under a stream of 5% CO_2 for 2 h. The adherent cells (macrophages) were separated from the nonadherent ones by washing, and 5×10^6 cells/ml of macrophages were pretreated with N-CWS at 37°C in a humidified atmosphere under a stream of 5% CO_2 . One hour later, N-CWS was removed by decanting the incubation medium, and the macrophages were washed with physiological saline, suspended again in HBSS without serum and cultured for 0.5, 1, 3, 4 or 24 h under the same conditions. The supernatant was separated by centrifugation at 1000 rpm for 10 min to assay L-929 cell-killing activity and fever-producing ability.

Gel Filtration Macrophages were pretreated with 100 $\mu\text{g}/\text{ml}$ of N-CWS for 1 h and cultured in HBSS without serum for 24 h after removing the N-CWS. The supernatant obtained was concentrated 6-fold by using a membrane filter (Amicon model 8200, YMS) and 2 ml of the solution was applied to a Sephadex G-200 column (i.d. $1.5 \times 82 \text{ cm}$); 2 ml fractions were collected by eluting with HBSS without serum and BSA at the rate of 4 ml/h at 4°C . Absorbance at 280 nm was spectrophotometrically measured for each fraction and 5 serial fractions were pooled for assay of L-929 cell-killing activity and fever-producing ability.

Assay of L-929 Cell-Killing Activity Killing of the L-929 cell line was used to measure the TNF-like activity of soluble factors essentially according to the method described by Fisch and Gifford.²⁰⁾ Serial 1:2 dilutions of the guinea pig sera samples, cultured supernatant of the macrophages or gel filtration fractions were placed in 96-well microtiter plates. Then 5×10^4 cells of L-929 were added to the microtiter plates in the presence of 2 $\mu\text{g}/\text{ml}$ of actinomycin D and 10% of fetal bovine serum (FBS). The cells were incubated at 37°C in a humidified atmosphere under a stream of 5% CO_2 for 18 h, and the medium was removed. The cells were stained with 0.5% crystal violet for 10 min and dried, and absorbance at 540 nm was measured. The cell-killing activity (unit) in the test samples was expressed as the reciprocal of the dilution which exhibited 50% cytotoxicity to the L-929 cells.

Results

In our previous studies,²¹⁾ N-CWS caused fever in guinea pigs at i.v. doses of 100 $\mu\text{g}/\text{kg}$ or more. To examine the relationship between the fever and TNF production, we used N-CWS at doses of 1 to 1000 $\mu\text{g}/\text{kg}$ in this study.

Pattern of Fever N-CWS induced fever at doses of more than 100 $\mu\text{g/kg}$. The fever appeared within 1–1.5 h and lasted about 4 h with peaks at 1.5 and 3–4 h (Fig. 1).

Fever and TNF-like Activity in the Sera N-CWS (1000 $\mu\text{g/kg}$ i.v.) was used in the experiment on TNF-like activity. Cytotoxic activity in the sera increased from 1 h after injection of N-CWS, peaked at 1.5 h, and returned to normal at 3 h (Fig. 2). On the other hand, fever appeared at 1.5 h and did not subside during the observation time. The fact that cytotoxic activity preceded the onset of fever suggests that a TNF-like mediator plays an important role as an EP in the first phase of fever induced by N-CWS, but not in the second phase or that from 2 h after the injection of N-CWS. Other factors such as IL-1 may participate in the latter phase of the fever.

Figure 3 shows the cytotoxic activity in the sera 1.5 h after an i.v. injection of 1–1000 $\mu\text{g/kg}$ of N-CWS. Cytotoxic activity could be detected in the sera of the animals given 100 and 1000 $\mu\text{g/kg}$ of N-CWS.

Production of TNF-like Cytokine in Guinea Pig Peritoneal Macrophages Treated with N-CWS

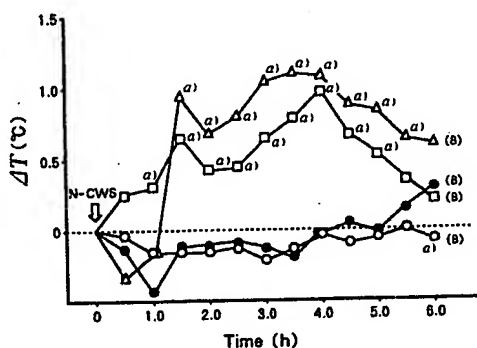


Fig. 1. Fever Pattern Induced in Guinea Pigs by N-CWS

A dose of 10 (○—○), 100 (□—□) or 1000 (△—△) $\mu\text{g/kg}$ of N-CWS, or physiological saline (●—●) was injected i.v. The arrow shows the time of injection. Values are expressed as mean \pm S.E. The numbers in parentheses are the number of guinea pigs used. Significantly different from the saline group, a) $p < 0.01$.

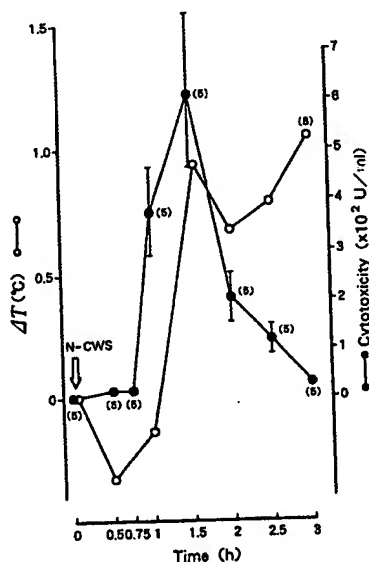


Fig. 2. Fever and Serum TNF-like Activity after i.v. Injection of N-CWS (1000 $\mu\text{g/kg}$) in Guinea Pigs

The arrow shows the time of injection. Values are expressed as mean \pm S.E. The numbers in parentheses are the numbers of guinea pigs used.

neal Macrophages Treated with N-CWS Since TNF is produced in macrophages,¹⁷⁾ we examined whether N-CWS would produce TNF in guinea pig peritoneal macrophages.

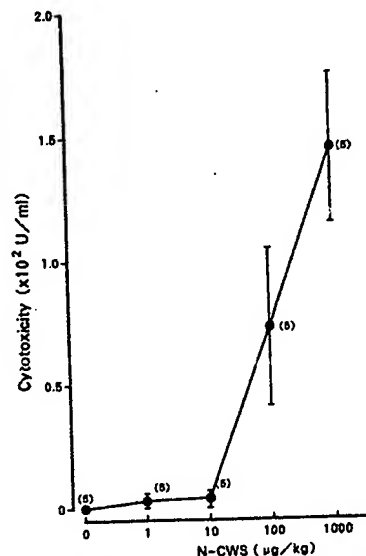


Fig. 3. Correlation between Serum TNF-like Activity and N-CWS Dose

TNF activity was measured 1.5 h after i.v. injection of N-CWS. Values are expressed as mean \pm S.E. The numbers in parentheses are the numbers of guinea pigs used.

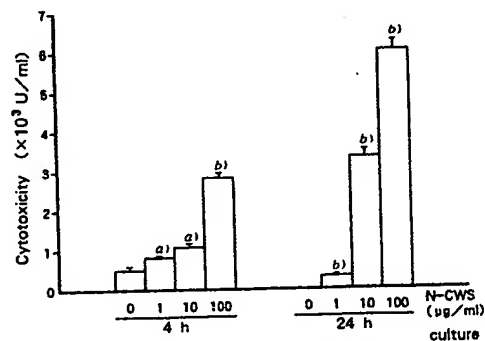


Fig. 4. TNF-like Activity in the Culture Supernatant of Guinea Pig Peritoneal Macrophages Pretreated with N-CWS

TNF-like activity was expressed as cytotoxicity of the culture supernatant to L-929 cells. Values are expressed as mean \pm S.E. Significantly different from the sample without N-CWS, a) $p < 0.05$, b) $p < 0.01$.

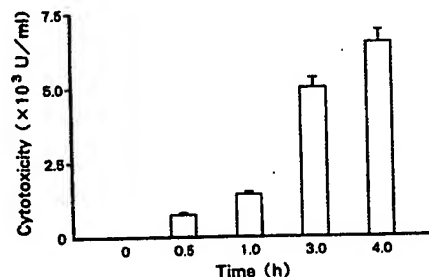


Fig. 5. TNF-like Activity in the Supernatant of Macrophages Cultured for a Short Time

Macrophages (5×10^6 cells/ml) were pretreated with 100 $\mu\text{g/ml}$ of N-CWS and cultured in HBSS without serum for 0.5–4.0 h after removing N-CWS. TNF-like activity was expressed as cytotoxicity of the culture supernatant to L-929 cells. Values are expressed as mean \pm S.E. TNF activity was not detected in the samples without N-CWS.

Macrophages were treated with N-CWS and cultured for 4 or 24 h. Cytotoxic activity was detected in all the supernatants of the macrophages treated with 1 $\mu\text{g}/\text{ml}$ or higher concentrations of N-CWS, and the activity was greater as the concentration was increased. Furthermore, the cytotoxic activity in the supernatants of the 24-h cultures was more than twice as strong as that of the 4-h cultures of macrophages treated with 10 or 100 $\mu\text{g}/\text{ml}$ of N-CWS. This suggests that cytotoxic activity also depends on culture duration (Fig. 4).

We propose that a TNF-like cytokine is produced in the macrophages immediately after treatment with N-CWS, because cytotoxic activity was detected in the supernatant of the macrophages as soon as 30 min after the start of incubation (Fig. 5).

Gel Filtration Pattern of the Cultured Supernatant In this study, 100 $\mu\text{g}/\text{ml}$ of N-CWS was used. When absorbance at 280 nm was measured for each fraction, 3 peaks of optical density (OD) were obtained, at fractions 27, 47 and 67. Cytotoxic activity was detected in fractions 41 to 55, and the greatest activity was in the pooled fractions 46 to 55. The molecular weight of materials in these pooled fractions was 32000–85000 as determined by gel filtration using molecular mass markers such as h- γ -G (Mol. wt. 160000), BSA (Mol. wt. 67000) and cyt-c (Mol. wt. 13000) (Fig. 6).

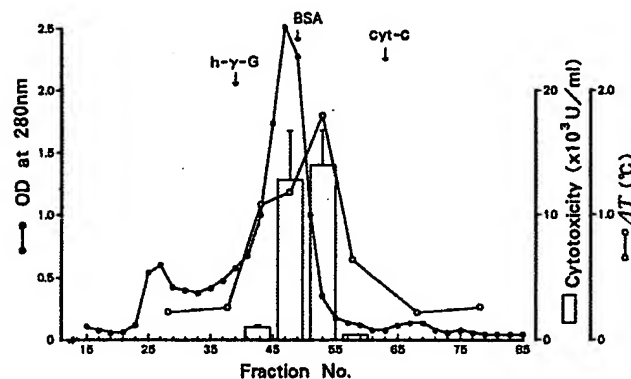


Fig. 6. Gel Filtration Pattern of the Culture Supernatant

The culture supernatant was applied to a Sephadex G-200 column (i.d. 1.5 \times 82 cm), and 2 ml fractions were collected by elution with HBSS. TNF-like activity and febrile response were measured for 5 pooled fractions. Febrile response was expressed as mean value of 3 guinea pigs 3 h after i.v. injection of 0.5 ml of the pooled fractions.

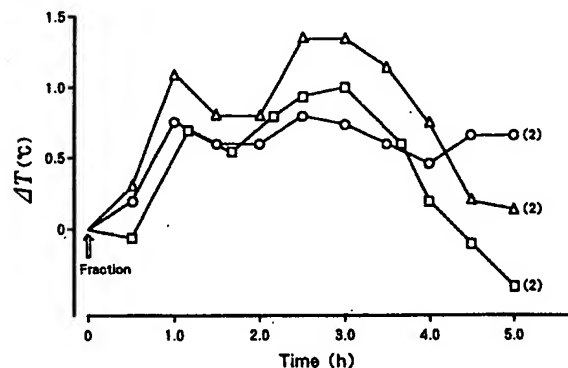


Fig. 7. Fever Response by Gel-Filtered Fractions

A dose of 0.1 (○—○), 0.5 (△—△) or 1.0 (□—□) ml of pooled fractions (fraction 46 to 50) was injected i.v. into guinea pigs. The arrow shows the time of injection. The numbers in parentheses are the numbers of guinea pigs used.

When 0.1, 0.5 or 1.0 ml of the pooled fractions 46 to 50 was injected i.v. into guinea pigs, biphasic fever with peaks at 1 and 2.5–3.0 h was observed in all the animals (Fig. 7). The pattern of the fever was similar to that induced by an injection of N-CWS, but its onset was 30 min earlier. In this case, the response was bell-shaped and the highest temperature was caused by 0.5 ml of the pooled fraction. In Fig. 6, febrile response 3 h after injection of 0.5 ml of each of the serially pooled fractions is shown. Clear fever of more than 0.5 $^{\circ}\text{C}$ was induced by fractions 41 to 60, and the highest fever was caused by the pooled fractions 51 to 55, in which cytotoxic activity was also greatest.

These results suggest that the fever induced by N-CWS is associated with TNF-like activity produced in the macrophages.

Discussion

For the reasons described below, it was suggested that TNF acted as an EP in the fever induced by N-CWS in guinea pigs. 1) TNF-like activity was detected in the sera 1 h after an injection of N-CWS and 30 min later, fever appeared. 2) When the guinea pig peritoneal macrophages were pretreated with N-CWS, increase of TNF-like activity in the culture supernatant depended on N-CWS concentration and culture duration. 3) The fraction with high TNF-like activity coincided with that with great fever-inducing ability, as determined by gel filtration.

Fever appeared within 1.5 h and lasted 5–6 h after an i.v. injection of N-CWS, whereas TNF-like activity appeared from around 1 h, peaked at 1.5 h, and then decreased. Dinarello *et al.*¹⁷⁾ reported that fever induced by an i.v. injection of r-TNF α occurred early and the febrile pattern was biphasic with peaks at 1 and 3–4 h in rabbits. They considered that TNF acted as an EP in the first phase of fever, but that in the second phase of fever, EPs other than TNF were involved, because more than 95% of r-TNF α injected in rabbits was cleared from the blood within 3 h.²²⁾ They suggested that the second phase was mediated by IL-1, because r-TNF α induced IL-1 in their experiment.¹⁷⁾ In our present study, the pattern of fever induced by N-CWS was similar to that induced by r-TNF α . Therefore, we propose that the first phase of fever was mediated by TNF produced by N-CWS, and that the EP on the second phase of fever would be IL-1, as suggested by Dinarello *et al.*, because the TNF induced by N-CWS disappeared quickly and was not detected in the second phase of fever in spite of this being higher. When macrophages were pretreated with N-CWS *in vitro*, the increase of TNF-like activity depended upon culture duration, and this pattern was quite different from that *in vivo*, in which TNF-like activity peaked 1 h after an injection of N-CWS and then quickly disappeared. We suggest that the cause of this difference lies in the clearance of TNF *in vivo*. When the supernatant of the cultured macrophages was gel-filtered, the fraction with TNF-like activity coincided with that with fever-inducing ability, and the patterns of the fever induced by the pooled fractions with TNF-like activity were similar to that of the fever induced by an injection of N-CWS. This may indicate mediation of the fever by TNF. The molecular weight of the pooled fractions with high TNF-like activity was determined to range from 32000–85000 by gel-filtration using molecular mass markers. Zacharchuk *et al.*²³⁾ report-

ed that the molecular weight of TNF from the sera, after challenge with an injection of LPS 14 d after the injection of *Bacillus Calmette-Guérin* into guinea pigs, was about 45000 as determined by high performance liquid chromatography (HPLC), and our data coincide well with this finding.

The results of the present experiments suggest that TNF acts as an EP in the fever induced by N-CWS, but the possibility that the first phase of the fever is mediated by other factors such as IL-1 cannot be excluded.

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**TUMOR NECROSIS FACTOR (CACHECTIN) IS AN
ENDOGENOUS PYROGEN AND INDUCES PRODUCTION OF
INTERLEUKIN 1**

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From the initial observations of Menkin and the pivotal experiments of Beeson, Wood and Atkins (1), the postulated mechanism for fever in a variety of diseases was based on the ability of various substances, usually of microbial origin, to stimulate phagocytes to synthesize and release a heat-labile protein called endogenous pyrogen (EP).¹ It was later shown that EP initiated fever by increasing prostaglandin PGE₂ synthesis in or near the anterior hypothalamus (reviewed in 2), and that antipyresis was the result of reduced cyclooxygenase activity there rather than in the EP-producing cells (3, 4). During the last decade, it became increasingly clear that EP, in addition to its ability to induce fever, possessed a great number of biological activities (5). Following tedious protein purifications, homogeneous EP was shown to stimulate T cells (6-9), increased hepatic acute-phase protein synthesis (10), activate neutrophils (11), stimulate prostaglandin production in vitro (12), and in general, mediate many components of the generalized acute-phase response (5). Because of its multiple biological activities, and particularly its ability to activate lymphocytes, renaming EP interleukin 1 has become accepted (13).

Attributing many diverse biological properties to a single molecule created a dilemma for investigators. Despite convincing evidence of the homogeneity of various preparations (8, 9, 14, 15), considerable doubt remained that a single polypeptide possessed such diverse activities. The controversy has now been resolved; two cDNAs coding for IL-1 have been cloned, a neutral form (16) and an acid form (17). Recombinant IL-1s of both forms have now been used to study the multiple biological properties attributed to IL-1. Although the two IL-1 forms, representing the two charged species at pI 7 and pI 5 (18), share little

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¹Abbreviations used in this paper: EP, endogenous pyrogen; LAL, limulus amoebocyte lysate; MNC, mononuclear cell; TNF, tumor necrosis factor.

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amino acid homology, each recombinant form can induce the same broad spectrum of responses, including fever (19).

During the early investigation into the pathogenesis of fever, there was speculation that another leukocyte product, interferon, also caused fever. From the first clinical trials using partially purified IFNs, fever had become the major side effect of IFN therapy (20). Current clinical trials using recombinant forms have confirmed the observations that IFNs, particularly IFN- α , cause fever. Recently (21, 22) it was shown that IFN- α produces fever not because it is contaminated with endotoxins or induces IL-1, but rather because IFN- α is intrinsically pyrogenic by its direct action on the thermoregulatory center (21, 22). Thus, from a conceptual point of view, a second molecule exists with endogenous pyrogen activity. rIFN- α meets the criteria for being an endogenous pyrogen: it produces a brisk, monophasic fever following intravenous injection into rabbits and mice, is free of endotoxins, and increases the production of PGE₂ from brain tissue in vitro and in vivo.

We now report that another leukocyte product, tumor necrosis factor (TNF: cachectin) (23), is also intrinsically pyrogenic. Recombinant human tumor necrosis factor (rTNF α) produces a brisk, monophasic fever after intravenous injection into rabbits, and the fever is not due to contaminating endotoxin. However, unlike IFN- α , rTNF α also induces IL-1 in vivo and in vitro. These results, along with those observed with IFN- α and IL-1, support an expanded hypothesis for the pathogenesis of fever involving several endogenous pyrogens.

Materials and Methods

Materials. Human rTNF α was expressed in *Escherichia coli* and purified to homogeneity (24). *Limulus* amoebocyte lysate (LAL) (Mallinckrodt, Inc., St. Louis, MO) testing employed an *E. coli* standard (Mallinckrodt, Inc.) and detected 10 pg/ml (0.1 endotoxin unit). Various lots of rTNF α were tested and revealed 200 pg or less of endotoxin per milligram of rTNF α . Adding rTNF α to known quantities of endotoxin did not interfere with the LAL assay. Using gas chromatography/mass spectrometry (5840A and 5985B, respectively, Hewlett-Packard Co., Palo Alto, CA), β -hydroxymyristic acid in rTNF α preparations was not detected above background levels (sterile water was <40 pg/mg). Adding known amounts of endotoxin to rTNF α resulted in complete recovery of the calculated amount of β -hydroxymyristic acid (25). The human rIL-1 used in these studies was the pl 7 form (16), provided by Cistron Technology, Inc. (Pine Brook, NJ). rIL-1 was expressed in *E. coli* and consisted of amino acids 112-269 of the precursor sequence. LAL testing used lysate from Associates of Cape Cod (Woods Hole, MA) with a sensitivity of 20 pg/ml (0.2 endotoxin unit) using the *E. coli* standard EC-5 (Bureau of Biologics, Bethesda, MD). The rIL-1 contained 40-80 pg endotoxin per milligram of protein. Adding rIL-1 to known quantities of endotoxin did not interfere with the LAL assay. Human rIFN- γ was produced in *E. coli* and purified to >98% purity (26). IFN- γ concentrations were determined in a cytopathic inhibition assay using A549 cells challenged with encephalomyocarditis virus. rIFN- γ used in these studies has a sp act of $1-2 \times 10^7$ U/mg. Ibuprofen formulated for intravenous injection was provided by Upjohn Co., Kalamazoo, MI.

Trypsin Treatment. rTNF α (2 mg) was incubated with TPCK-treated trypsin (Cooper Biomedical, Freehold, NJ) at 37°C. Trypsin (20 μ g) was added at 0, 12, 24, and 36 h. After 48 h of incubation, soybean trypsin inhibitor (160 μ g; Sigma Chemical Co., St. Louis, MO) was added to each sample. *Limulus* lysate testing of the trypsinized rTNF α revealed 1 ng/mg endotoxin.

Pyrogen Testing. New Zealand-derived female rabbits weighing ~2.5-3.0 kg (Pine

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Acres, Burlington, VT) were trained in restraining devices for 1 wk before pyrogen testing. Core temperature was measured using indwelling rectal thermistors (Yellow Springs Instruments, Yellow Springs, OH) and a Kaye Digistrip Model II recorder (Bedford, MA). Rectal temperatures were measured every minute, as described previously (21). The murine fever studies used C3H/HeJ mice, individually caged in an ambient temperature of 33–34°C. Body temperatures were measured with thermosensitive radiotelemetry devices (XM-FH; Minimitter Co., Inc., Sunriver, OR) implanted subcutaneously. After 2 h of stabilization, each mouse was injected with 0.1 ml of either PBS or a dilution of rTNF in PBS (six mice per group). Body temperature was measured at 10-min intervals before and after injection by determining the frequency emitted by each telemeter. The signal was detected with a Minimitter RA-1000-TH receiver coupled to a digital frequency counter (Heathkit SM 2420, Benton Harbor, MI) and converted to temperature using a previously determined calibration index.

PGE₂ Production and Measurements. Rabbit hypothalami were removed, minced, and incubated for 15 min at 37°C. After washing to remove PG induced by tissue trauma, aliquots were incubated for 60 min with various concentrations of rTNF α or rIL-1 in the presence of clinical grade polymyxin B (25 μ g/ml) (Pfizer, Groton, CT) as previously described (21, 28). The supernatants were frozen at -70°C and later assayed for PGE₂ using a specific RIA (Seragen, Boston, MA).

Human Mononuclear Cell Incubations. Blood was obtained from healthy donors, and the mononuclear cells (MNC) were isolated as previously described (29). Methods for stimulating endogenous pyrogen activity for rabbit pyrogen testing using 5-ml volumes of MEM (Microbiological Associates, Walkersville, MD) have been described in detail (29). MNCs were also incubated with IFN- γ and rTNF α for 48 h in 1-ml wells with 2.5×10^6 cells/ml. For these experiments, MNCs were suspended in MEM (Gibco, Grand Island, NY) containing 10% FCS (Hyclone, Logan, UT). Supernatants were diluted and assayed for IL-1 activity on thymocytes.

IL-1 Murine Thymocyte Assay. Thymocytes from C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME) were removed and used to test IL-1 activity in the supernatants of human MNC using augmentation of [³H]thymidine incorporation in response to suboptimal concentrations (1 μ g/ml) of PHA-P (Burroughs Wellcome, Research Triangle, NC) as previously described (9, 30). Purified human monocyte IL-1 was used as a standard in these assays (9).

Western Blot Analysis. Methods for western blot analysis were used as previously described (37). Blots were developed using goat anti-rabbit IgG followed by ¹²⁵I-labeled protein A (Amersham Corp., Arlington Heights, IL).

Cytotoxicity Assay. Cytotoxicity of the murine fibroblast L929 line was used to assay rTNF α activity (31).

Results

Pyrogenicity of rTNF α . Rabbits were given an intravenous bolus injection of freshly thawed rTNF α diluted and mixed with 1.0 ml of 0.15 M NaCl containing 250 μ g/ml of polymyxin B. As shown in Fig. 1A, rTNF α and rIL-1 at a dose of 1 μ g/kg produce febrile responses characteristic of endogenous pyrogens, with monophasic fevers reaching mean peak elevations 48–54 min after the injections. The injection of 0.1 μ g/kg of rTNF α did not induce significant fever (<0.3°C), whereas 0.1 μ g/kg of rIL-1 induced small but significant fever (>0.3°C; data not shown). The fevers produced by either rTNF α or rIL-1 are unlike those produced by endotoxin in which the monophasic fever reaches peak elevation at ~90 min after the injection (1). In addition, the amount of endotoxin that was detected in either the rTNF α or rIL-1 was clearly below the minimum pyrogenic threshold for endotoxins in rabbits (3–5 ng/kg) (1). As shown in Fig. 1B, the fever induced by rTNF α is blocked by prior treatment with the cyclooxygenase

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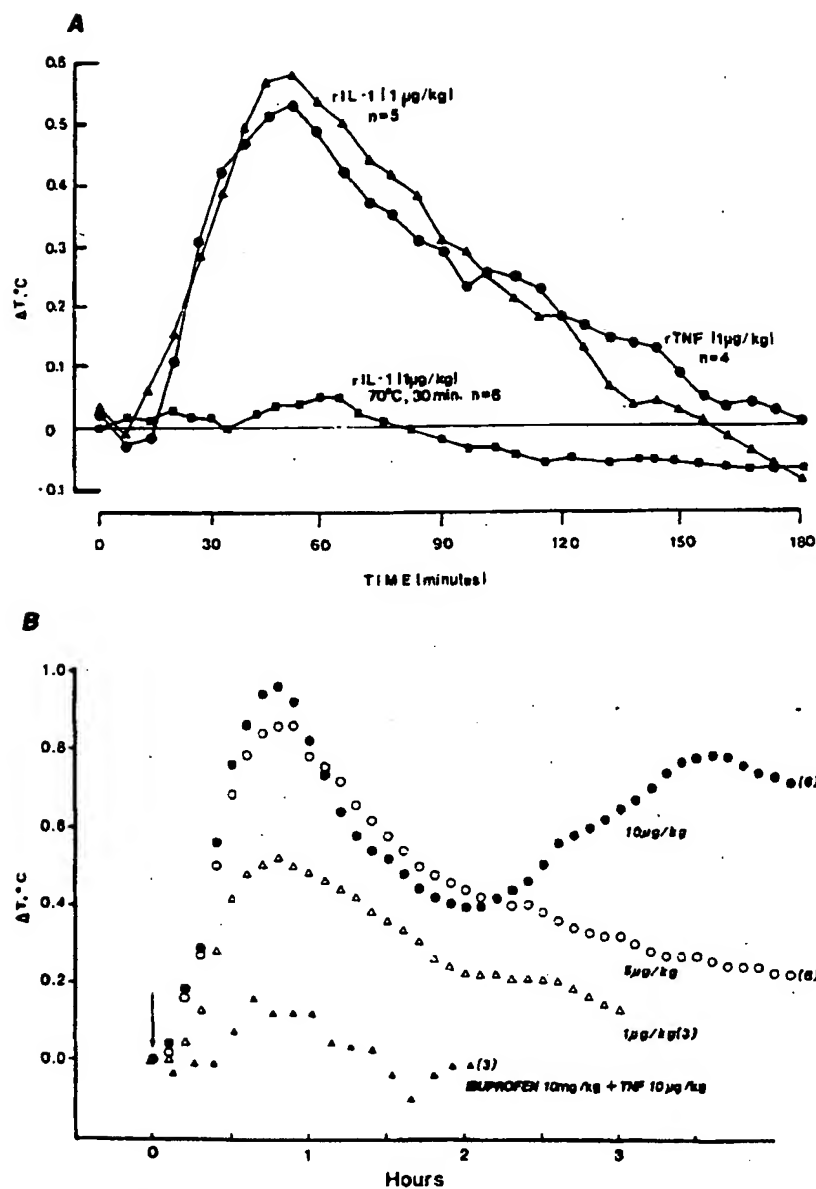


FIGURE 1. (A) Mean fevers of rabbits injected with either rTNF α or rIL-1. The recombinant materials were thawed, diluted in polymyxin B and injected intravenously. The numbers in parentheses indicate the number of rabbits in each group. (B) Dose-response of rTNF α in rabbits. rTNF α was treated as indicated in A. Ibuprofen was injected intravenously 10 min before rTNF α .

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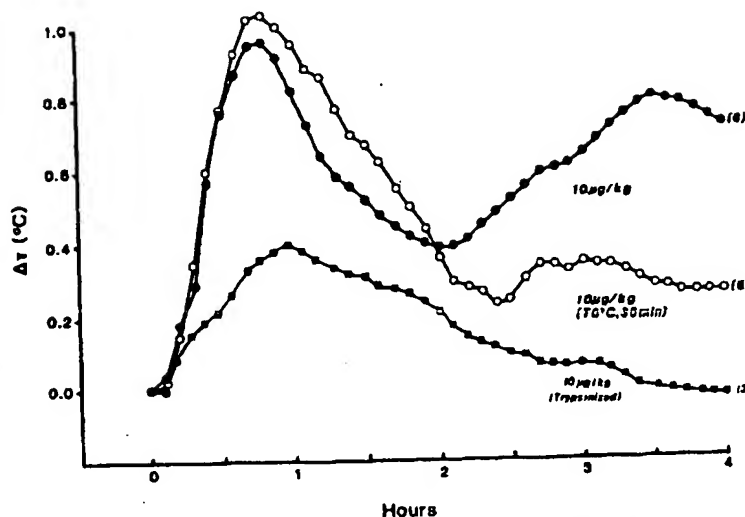


FIGURE 2. Mean fevers in rabbits injected with rTNF α (10 μ g/kg) treated with various conditions, as indicated. The numbers in parentheses represent the number of rabbits in each group.

inhibitor and antipyretic, ibuprofen (32). Higher doses of rTNF α (10 μ g/kg) induce biphasic fevers with the second fever peak occurring after 3.5 h. As shown in Fig. 2, heating rTNF α for 30 min at 70°C reduced the second fever peak without affecting the initial febrile response. However, the pyrogenicity of rTNF α was markedly reduced by trypsin treatment. The cytotoxicity assay of trypsinized rTNF α revealed that the treatment reduced biological activity of the rTNF α by ~80% and that heat treatment reduced activity by 50% (data not shown).

Although the concentrations of endotoxin in the rTNF α (see Materials and Methods) were below that which would induce fever in rabbits, we employed the endotoxin-resistant C3H/HeJ mouse for additional pyrogen testing. As shown in Fig. 3, rTNF α produces fever in these mice at 1 and 10 μ g/kg. Significant elevations of body temperature occurred 40 min after intraperitoneal injections.

Rabbits injected daily with endotoxins develop progressive pyrogenic unresponsiveness (pyrogenic tolerance). In an attempt to induce pyrogenic tolerance to rTNF α , injections of rTNF α (10 μ g/kg) were given to rabbits on four consecutive days. In general, no demonstrable decreases in febrile responses were observed. Three representative individual rabbit febrile responses are shown in Fig. 4. Rabbit 2 did show decreasing fever with successive injections of rTNF. However, this rabbit became ill (diarrhea and lethargy) after the second injection and died before the fourth injection. During the course of these studies, 56 rabbits received repeated injections of rTNF α (all intravenous), and 6 rabbits (11%) were found dead in their cages. In contrast, no rabbits receiving repeated injections of rIL-1 died.

To determine the cause of the second fever peak, rabbits injected with rTNF α were bled during the uprise of their second fever peak, and the heparinized

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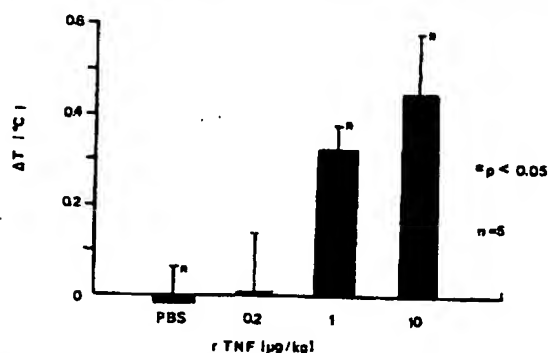


FIGURE 3. Mean peak fever (\pm SEM) after 40 min in C3H/HeJ mice injected with materials indicated. Significance was reached between the PBS control and the rTNF α as shown using analysis of variance.

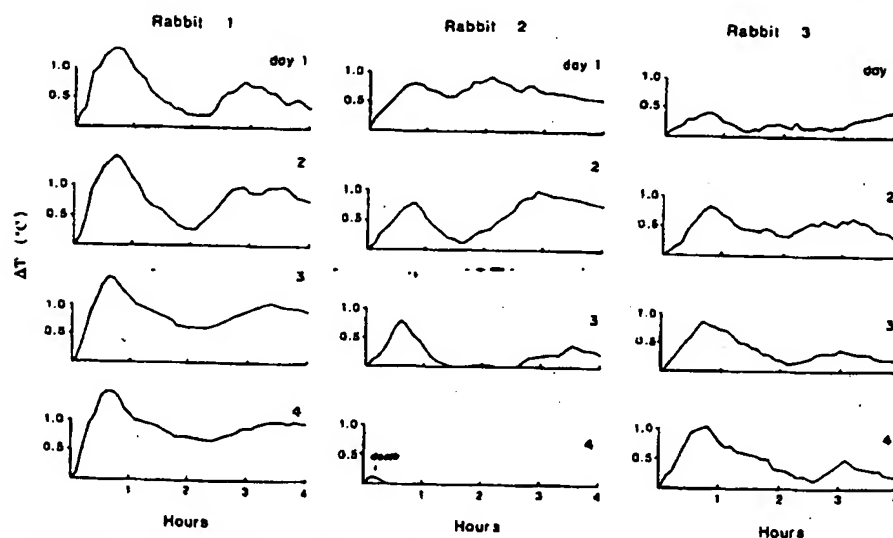


FIGURE 4. Febrile responses to four daily injections of rTNF α (10 μ g/kg) in three rabbits.

plasma was pooled and stored for 24 h at 4°C. This plasma was injected into fresh rabbits, and as shown in Fig. 5, produced a monophasic fever. The pyrogenicity of the circulating plasma was destroyed by heating at 70°C for 30 min. The heat lability of the plasma factor is similar to that of rIL-1, as shown in Fig. 1A. On the other hand, rTNF α , heated in the same water bath as the rIL-1, retained its ability to induce the first fever peak (see Fig. 2). The data from these experiments support the concept that the second fever peak after rTNF α is due to the production of IL-1 *in vivo*.

rTNF α and rIL-1 Induce Hypothalamic PGE $_2$ *In Vitro*. The rapid rise in body temperature that characterizes the febrile response to either rTNF α or rIL-1 suggests a direct hypothalamic stimulation, most likely mediated, in part, by an increase in PGE $_2$ synthesis. A single intravenous injection of ibuprofen (10 mg/kg) given immediately before rTNF α blocked the febrile response, as pre-

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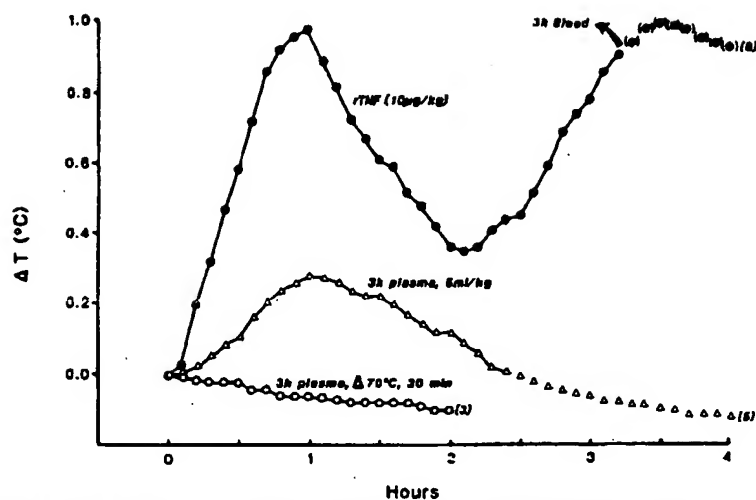


FIGURE 5. Mean fevers in six rabbits injected with materials indicated. After 3 h, three rabbits were bled via the central ear artery. The plasma was pooled, stored at 4°C and warmed to 37°C before administration the next day. This plasma was infused intravenously over 2 min.

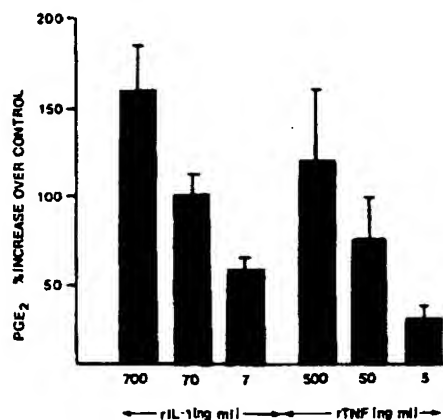


FIGURE 6. Mean percent increase of PGE_2 as measured in supernatants of stimulated rabbit hypothalamic minces. Unstimulated cells produce 30–60 pg/mg of tissue.

viously shown for IL-1 (32). Rabbit hypothalamic minces were incubated with $rTNF\alpha$ or $rIL-1$ for 60 min, and the level of PGE_2 was assayed in the supernatant media. As reported previously (12, 21, 28), one onehundredth of the amount of IL-1 necessary to produce 0.6°C fever in rabbits approximately doubled the PGE_2 detected in the supernatant of hypothalamic minces after 60 min. As shown in Fig. 6, the specific activities of $rTNF\alpha$ and $rIL-1$ were similar in terms of PGE_2 production in three separate experiments.

Production of IL-1 In Vitro from Human MNC Incubated with $rTNF\alpha$. To elucidate the probable mechanism by which intravenous injection of $rTNF\alpha$ in rabbits induces the production of a circulating endogenous pyrogen (IL-1 or

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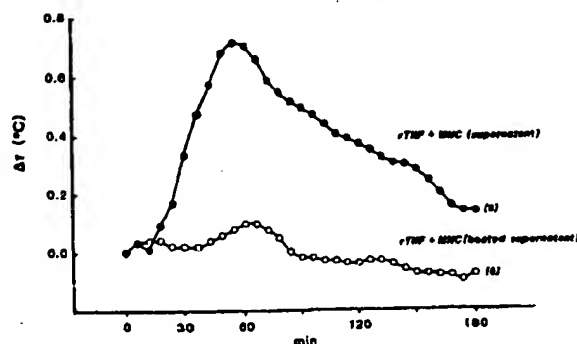


FIGURE 7. Mean fevers in rabbits injected with supernatants derived from human MNC stimulated with rTNF α (50 ng/ml). Supernatants from the MNC of two human subjects were pooled; half was heated (70°C for 30 min) before injection. Rabbits were injected with supernatants derived from 10^6 monocytes/kg. The numbers in parentheses indicate the number of rabbits used to assay the supernatants.

TABLE I
Neutralization of Pyrogenic Activity in MNC Supernatants by
Anti-IL-1

MNC supernatants* plus:	Mean temperature peak (°C) [†]
Normal rabbit serum [‡]	0.64 \pm 0.08
Anti-human IL-1 [‡]	0.17 \pm 0.03

* 24-h MNC supernatants were generated in 25-ml flasks containing 5×10^6 MNC/ml (5 ml/flask) in the presence of 100 ng/ml rTNF α . For these experiments, MNC were isolated from two human subjects. Rabbits were injected with the supernatant derived from 10^6 monocytes/kg.

[‡] Anti-IL-1 (15) or normal rabbit serum was mixed at 1% (vol/vol) with the MNC supernatants, incubated overnight at 4°C and centrifuged (10,000 g) for 2 min.

[†] Number of rabbits used to assay supernatants.

TNF), human MNC were stimulated in vitro with various concentrations of rTNF α , and the supernatant media were assayed for endogenous pyrogen activity in rabbits. As shown in Fig. 7, the supernatant medium from human MNC incubated for 24 h with rTNF α induced a monophasic fever when injected into rabbits. The amount of rTNF α added to the MNC (50 ng/ml) was below the rabbit pyrogen threshold (see Fig. 1B). After the incubations, the supernatant media were tested for TNF α activity using an ELISA, and there was no increased TNF α in the supernatant over the amount that was added exogenously (data not shown). The pyrogenic activity in these supernatants was destroyed by heating at 70°C for 30 min, suggesting that this was likely due to IL-1 and not monocyte-derived TNF/cachectin. As shown in Table I, the monophasic fever-inducing activity in the stimulated MNC supernatants was IL-1, since it was neutralized by anti-human monocyte IL-1. This antibody did not recognize human rTNF or naturally-derived cachectin, as determined by western blot analysis (Fig. 8). Anti-IL-1 had no effect on rTNF α in the cytotoxicity assay (data not shown).

The dose response of rTNF α induction of IL-1 is shown in Fig. 9. At

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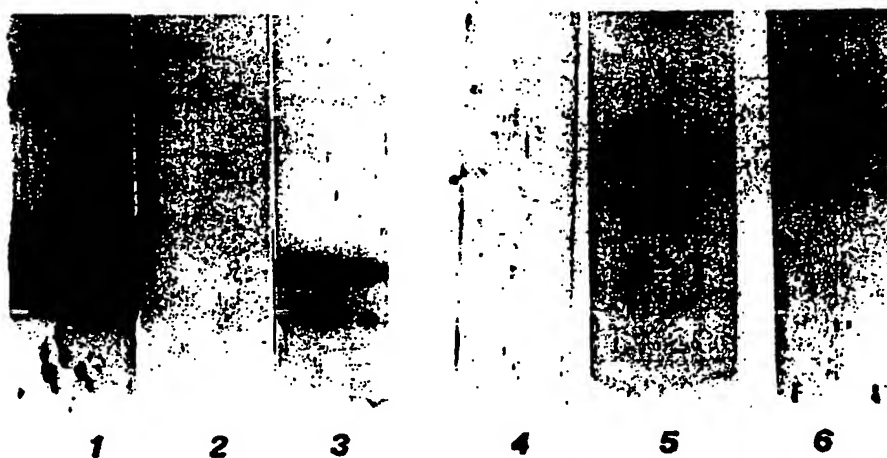


FIGURE 8. Western blot analysis of anti-IL-1. Lanes 1-3 each contain 1 µg purified natural cachectin (37). Lane 1 was developed with anticachectin (1:200); lane 2 with anti-IL-1 (1:100) (15); and lane 3 with anti-human rTNFα (1:200). Lanes 4-6 each contain 10 µg human rIL-1. Lane 4 was developed with anticachectin; lane 5 with anti-human IL-1; and lane 6 with anti-rTNFα.

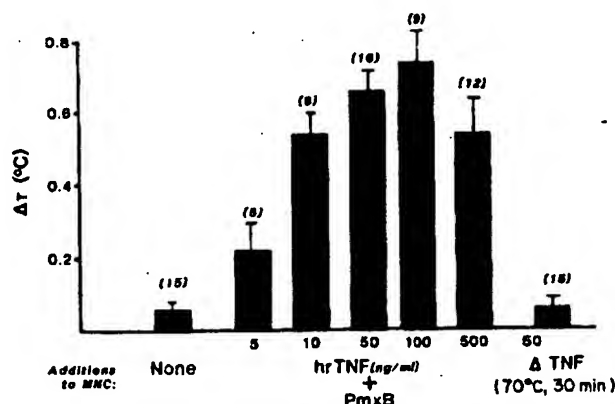


FIGURE 9. Mean peak fever ± SEM in rabbits injected with materials indicated under the abscissa. All MNC incubations were in the presence of 12.5 µg/ml polymyxin B. The numbers in parentheses indicate the number of rabbits used to assay the pyrogenic activity in the MNC supernatants.

concentrations of 10-500 ng/ml, rTNF induced IL-1 (as measured by peak fever in rabbits). The amount of rTNFα that induced IL-1 was optimal between 50 and 100 ng/ml, whereas concentrations of 500 ng/ml induced less IL-1 (although not significantly different from 100 ng/ml, $p > 0.05$). Heating rTNFα for 30 min at 70°C destroyed its ability to induce IL-1 in vitro; this finding confirms the observation that heat-treated rTNFα does not induce a second fever peak in rabbits (see Fig. 2).

As expected, supernatants of human MNC stimulated with rTNFα contained IL-1, as measured by augmentation of T cell proliferation in response to PHA (Table II). The IL-1-inducing property of rTNFα was sensitive to heat and

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TABLE II
Production of IL-1 by Human MNC In Vitro

Additions to MNC	[³ H]TdR incorporation (cpm)*			
	Donor 1	Donor 2	Donor 3	Donor 4
None (MNC control)	6,763	4,711	5,096	4,793
rTNF α (ng/ml)				
1	35,049	10,212	13,542	ND
10	48,924	20,118	8,966	ND
100	24,864	9,426	7,254	10,471
100; trypsinized	ND	ND	ND	4,470
Trypsin only	ND	ND	ND	6,086
Endotoxin (10 ng/ml)	125,864	33,824	64,357	ND

* Mean counts per minute of [³H]TdR incorporation. SD of triplicate wells were 5–10% of the mean. MNC supernatants were diluted 1:20 and 1:200 in the thymocyte assay. Only 1:20 data are shown.

trypsinization. However, the amount of IL-1 detected in the T cell assay was less than that assayed in endotoxin-stimulated MNC supernatants. This may have been due to interference of TNF α in the IL-1 assay, although incubating rIL-1 with rTNF α in the thymocyte assay resulted in only slight inhibition of the IL-1 activity (data not shown). It is also possible that rTNF α stimulated the production of inhibitory substances from the mononuclear cells. Although detection of these IL-1-inhibiting substances in the rabbit pyrogen assay is unlikely, several investigators (33) have shown that stimulated monocytes release substances that inhibit T cell proliferation in this assay.

Production of IL-1 from human MNC by rTNF α was enhanced by coinubation with rIFN- γ . As depicted in Fig. 10, rIFN- γ (1 ng/ml) enhanced IL-1 production by rTNF α at 0.25 ng/ml.

rTNF α has no intrinsic proliferation-enhancing activity except at high concentrations (500 ng/ml; $p < 0.05$) (Fig. 11). This activity may reflect the induction of IL-1 from contaminating murine thymic epithelial cells or macrophages, since it was neutralized with anti-mouse IL-1. In situ production of IL-1 during the T cell assay has been observed with other substances, such as the polypeptide toxin elaborated by certain strains of toxic shock syndrome-associated *Staphylococcus aureus* (34).

Discussion

These studies show that rTNF α causes fever because it is intrinsically pyrogenic and is capable of increasing hypothalamic PGE₂ synthesis. Following an intravenous injection of 1 μ g/kg, rabbits develop monophasic fevers that reach peak elevations after 45–55 min and then return to baseline levels. The only other well-defined substance of leukocyte origin that produces this rapid increase in core temperature is IL-1. Because it is a product of stimulated leukocytes, TNF/cachectin must now be considered an endogenous pyrogen. In Phase I clinical trials, rTNF α injected into humans is pyrogenic (S. Sherwin and S. Saks, unpublished observations). Mice passively immunized with anticachectin antibodies survive a lethal dose of endotoxin but still develop fever (35). In these mice

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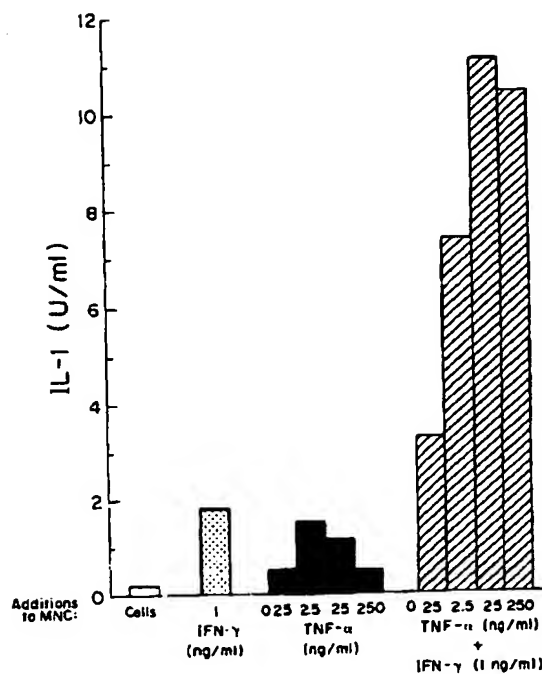


FIGURE 10. IL-1 production by rTNF α and enhancement by rIFN- γ . MNC supernatants were incubated in the presence of 10% FCS for 48 h with materials indicated under the abscissa and diluted 1:8 in the thymocyte assay.

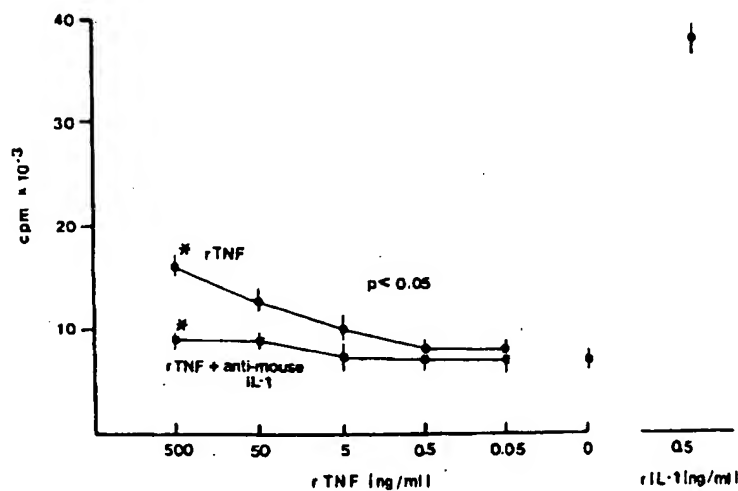


FIGURE 11. Comparison of rTNF α and rIL-1 on thymocyte proliferation to PHA. Anti-mouse IL-1 (1:2,000 dilution, vol/vol) was added to some wells with the rTNF α . Anti-mouse IL-1 was raised in goats to IL-1 purified from P388D cell line (kind gift of Dr. Morris Sheetz, Eli Lilly and Co., Indianapolis, IN). With these thymocytes, concentrations of rTNF α at 500 ng/ml induced significant IL-1-like activity ($p < 0.05$, Student's *t*-test.).

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it is likely that both cachectin and IL-1 are produced, but IL-1 is not neutralized by the anticachectin antibody. Thus, it is IL-1 that probably mediates the fever in these passively immunized mice. It appears that rTNF α and rIL-1 have approximately the same specific activities in terms of their ability to induce rapid-onset fevers after intravenous injection in mice and rabbits. At present, there are no studies of rIL-1 injected into human subjects, and the sensitivity of humans to IL-1 remains to be studied. Although there is another endogenous pyrogen, IFN- α (21, 22), the amount of IFN- α required to produce fever in rabbits or mice is 100-fold greater than rIL-1 or rTNF α . However, IFNs have known species specificities which may explain this dose-relationship in nonhuman species.

From clinical and laboratory data, three leukocyte products can now be identified as being endogenous pyrogens, IL-1, IFN- α and TNF/cachectin. What remains to be established is whether any or one or all three of these substances mediates the febrile response to a particular disease. In patients with infectious diseases in which endotoxin plays a pathogenic role, it is likely that all are produced. Extensive laboratory evidence has established that endotoxin stimulates TNF/cachectin production (36, 37), IL-1 (13), and IFN production (38, 39). However, it may be that in some viral diseases, i.e., cytomegalic disease and hepatitis B, IFN predominates as the primary endogenous pyrogen, since many components of the acute-phase response are not induced by IFN and are frequently absent in various viral diseases. Likewise, TNF/cachectin may be the mediator of fever in certain parasitic diseases. Considerable clinical and laboratory investigation remain before the role of each or combinations of these endogenous pyrogens can be established in a comprehensive explanation for the pathogenesis of fever.

The recently reported amino acid sequences of human TNF- α (24), cachectin (23) and IL-1 (16, 40) indicate no obvious sequence homologies. Two forms of IL-1 have been identified (16, 17), which correspond to the two isoelectric points, pI 7 and 5. These two forms were originally described as endogenous pyrogens in 1974 (18), but their physical relationship at that time was not clear. At the amino acid level, these two forms share little homology; however, at the carboxyl end of each form, 38% absolute and 78% conserved sequence homology exist (41), and it seems likely that the active site for the biological properties which the two IL-1 forms share resides in these sequences. Nevertheless, when the shared carboxyl segments are matched with TNF α or lymphotoxin (TNF β), no striking sequence homology can be observed. Despite their independent structure, TNF/cachectin and IL-1 both produce fever, stimulate neutrophils (11, 42), induce synovial cell collagenase production (43), decrease lipoprotein lipase (37, 44), and are cytotoxic for tumor cells (45, 46).

Using rTNF α in these studies, as has been shown (37) using purified natural cachectin, TNF/cachectin has no IL-1 activity on T cells. This may explain the findings of Damais et al. (47), who reported that muramyl dipeptide polyalanine/lysine stimulated macrophages to release a pyrogenic substance that is not active on lymphocytes. Induction of TNF/cachectin would explain the activity responsible for the fever-producing property of these supernatants.

At higher doses, rTNF α induces a biphasic fever. Using passive transfer of

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plasma taken during the rise of the second fever peak, a circulating substance produced a monophasic fever when injected into new rabbits. This circulating pyrogenic substance is probably not residual rTNF α , since the pharmacokinetics of rTNF and natural cachectin indicate that >95% of the injected material is cleared from the circulation in 3 h (48). The experiments presented in the present study suggest that the second fever peak represents IL-1 induced by rTNF α in vivo. To support this observation, rTNF α was shown to induce IL-1 production from human MNC in vitro. Although the rabbit pyrogen assay was used to test the IL-1 content of the stimulated MNC supernatants, it can no longer be assumed that brisk monophasic fevers in rabbits is a valid assay for the presence of IL-1. As a result of these studies, it is now clear that either rTNF α or rIL-1 induce indistinguishable fevers within 60 min of intravenous injection. Thus, the endogenous pyrogen assay in rabbits requires conditions for specificity. At present, these seem to be (a) the ability of heating at 70°C for 30 min to destroy the fever-inducing property of IL-1 but not rTNF α , and (b) the use of an anti-human IL-1, which neutralizes IL-1's pyrogenic property but does not recognize TNF/cachectin. Using these two conditions, it seems clear that the pyrogenic moiety in rTNF α -stimulated MNC supernatants is indeed IL-1 and not more TNF. The use of the T cell assay was also important in demonstrating that rTNF α induces IL-1, since TNF/cachectin is not active on T cells.

The biphasic fever curve seen after administration of rTNF α suggests that rTNF α has two separate effects: the induction of PGE₂ and the induction of IL-1. The initial fever peak (PGE₂-mediated) has a much lower threshold (<1 μ g/kg) than the secondary fever peak (IL-1-mediated) in which the dose of rTNF α must be between 5 and 10 μ g/kg in order to evoke the delayed fever (Fig. 1B). In addition, the primary response is not abolished by prior heating of rTNF α at 70°C for 30 min, whereas the secondary response is completely eliminated by this treatment. However, based on the cytotoxicity assay, the heat-treated rTNF α still retains 20% of its original activity. The loss of the secondary fever response probably reflects this decrease in potency. Currently, we have no evidence for the presence of two distinct receptors mediating these two effects for rTNF α , but it seems unlikely that a single receptor would have two different thresholds, each mediating a separate response such as the production of PGE₂ and IL-1.

Previous work (49) has shown that colony-stimulating factors induce IL-1 production. In addition, there are partially characterized lymphokines, secreted in response to mitogen or antigen, which induce monocytes to produce IL-1 (50-52). The present studies raise the question of whether one of these lymphokines is lymphotoxin (TNF β), since this molecule is closely related to TNF/cachectin (24).

These studies shed light on the long-unexplained finding (53-55) that during the second fever peak in rabbits given intravenous injections of influenza virus, the plasma contained a substance which, when injected into new rabbits, induced another biphasic fever. It had been established that the pyrogenic moiety circulating during the second fever peak was not due to remaining virus but rather to a host product. It was also shown that fever-inducing factor(s) circulating during the plasma-induced second fever peak contained characteristic endogenous pyrogen (54), and it now seems certain that this pyrogen was IL-1. However,

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using large amounts of purified rabbit IL-1, only monophasic fevers have been observed (P. Murphy, personal communication). Thus, the concept that IL-1 induces more IL-1 in vivo has been difficult to substantiate even using in vitro production methods (55). Considering the data of the present report, it seems possible that the circulating factor detected by passive transfer of plasma from febrile rabbits may have been due to TNF/cachectin rather than IL-1.

Summary

Recombinant human tumor necrosis factor (rTNF α) injected intravenously into rabbits produces a rapid-onset, monophasic fever indistinguishable from the fever produced by rIL-1. On a weight basis (1 μ g/kg) rTNF α and rIL-1 produce the same amount of fever and induce comparable levels of PGE₂ in rabbit hypothalamic cells in vitro; like IL-1, TNF fever is blocked by drugs that inhibit cyclooxygenase. At higher doses (10 μ g/kg) rTNF α produces biphasic fevers. The first fever reaches peak elevation 45–55 min after bolus injection and likely represents a direct action on the thermoregulatory center. During the second fever peak (3 h later), a circulating endogenous pyrogen can be shown present using passive transfer of plasma into fresh rabbits. This likely represents the in vivo induction of IL-1. In vitro, rTNF α induces the release of IL-1 activity from human mononuclear cells with maximal production observed at 50–100 ng/ml of rTNF α . In addition, rTNF α and rIFN- γ have a synergistic effect on IL-1 production. The biological activity of rTNF α could be distinguished from IL-1 in three ways: (a) the monophasic pyrogenic activity of rIL-1 was destroyed at 70°C, whereas rTNF α remained active; (b) anti-IL-1 neutralized IL-1 but did not recognize rTNF α or natural cachectin nor neutralize its cytotoxic effect; and (c) unlike IL-1, rTNF α was not active in the mitogen-stimulated T cell proliferation assay. The possibility that endotoxin was responsible for rTNF α fever and/or the induction of IL-1 was ruled-out in several studies: (a) rTNF α produced fever in the endotoxin-resistant C3H/HeJ mice; (b) the IL-1-inducing property of rTNF α was destroyed either by heat (70°C) or trypsinization, and was unaffected by polymyxin B; (c) pyrogenic tolerance to daily injections of rTNF α did not occur; (d) levels of endotoxin, as determined in the *Limulus* amoebocyte lysate, were below the minimum rabbit pyrogen dose; and (e) these levels of endotoxin were confirmed by gas chromatography/mass spectrometry analysis for the presence of β -hydroxymyristic acid. Although rTNF α is not active in T cell proliferation assays, it may mimic IL-1 in a T cell assay, since high concentrations of rTNF α induced IL-1 from epithelial or macrophagic cells in the thymocyte preparations. These studies show that TNF (cachectin) is another endogenous pyrogen which, like IL-1 and IFN- α , directly stimulate hypothalamic PGE₂ synthesis. In addition, rTNF α is an endogenous inducer of IL-1. Together, these results support the concept that the febrile response to infection is a fundamental event in host defense and the induction of fever by endogenously produced molecules is not imparted to a single substance.

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Characteristics of fever and acute-phase response induced in rabbits by IL-1 and TNF

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MORIMOTO, AKIO, YOSHIYUKI SAKATA, TATSUO WATANABE, AND NAOTOSHI MURAKAMI. *Characteristics of fever and acute-phase response induced in rabbits by IL-1 and TNF*. Am. J. Physiol. 256 (Regulatory Integrative Comp. Physiol. 25): R35-R41, 1989.—We investigated the effect of human recombinant interleukin 1 α (hrIL-1 α) and human recombinant tumor necrosis factor (hrTNF) on body temperature and acute-phase response, including changes in the plasma concentration of iron, zinc, and copper and in circulating leukocyte count. The intravenous injection of a smaller dose of either hrIL-1 α (0.5 μ g/kg) or hrTNF (2 μ g/kg) produced a monophasic fever, whereas a larger dose (hrIL-1 α , 2 μ g/kg; hrTNF, 10 μ g/kg) produced a biphasic fever. The intracerebroventricular injection of hrIL-1 α or hrTNF produced a dose-dependent fever. The intravenous injection of either hrIL-1 α or hrTNF decreased the plasma concentration of iron and zinc and increased the plasma copper concentration and the circulating leukocyte count. The intracerebroventricular injection of hrIL-1 α induced those responses, although the intracerebroventricular injection of hrTNF did not. The present results show that two kinds of monokines, hrIL-1 α and hrTNF, are intrinsically pyrogenic and induce the acute-phase response. Furthermore, it is suggested that hrIL-1 α induces febrile and acute-phase responses through its action on both the peripheral target organs and the central nervous system. However, hrTNF induces those responses only by its action on the peripheral target organs outside the blood-brain barrier.

thermoregulation; pyrogen; monokine; cytokine

IT HAS BEEN GENERALLY believed that an exogenous pyrogen, such as an endotoxin of bacteria, causes fever by inducing circulating and reticuloendothelial monocytes to synthesize and release a small mediator protein called endogenous pyrogen (EP) (2). Subsequently EP raises body temperature by acting on the central nervous system (CNS). Furthermore, increasing evidence has revealed that EP, in addition to its ability to produce fever, induces changes in the plasma level of certain trace metals, activation of hepatic protein synthesis, and an increase in circulating leukocyte count that are termed collectively the acute-phase response (18, 19, 21). Now, it is generally recognized that fever and acute-phase response represent a primary host defense response (23, 24).

During the last decade, EP has been so highly purified that its amino acid sequences have been determined (3), and the renaming of EP as interleukin 1 (IL-1) has been

commonly accepted (10). Furthermore, since the cDNA coding for IL-1 has been cloned (27), recombinant IL-1 has now been used to study the genesis of fever and acute-phase response. However, in recent advances of research in immunology, several kinds of cytokines released from various kinds of leukocytes and other cells have been found to constitute part of the immunoregulatory networks for host defense. These cytokines are also small mediator proteins with molecular weight similar to IL-1, ranging from 15,000 to 30,000, despite no obvious sequence homologies. Among them, it has been recently shown that interferons released from lymphocytes induce fever (13, 30). Moreover, tumor necrosis factor (TNF; cachectin), one of the monokines, as well as IL-1 (4), has been found to be intrinsically pyrogenic (5, 8, 14, 34). Therefore it is considered that naturally occurring fever is mediated not only by IL-1 but also by several kinds of cytokines. Now we must take into account the possibility that febrile response depends on the relative contribution of each kind of cytokine under different pathological conditions.

As for the mechanisms of induction of febrile and acute-phase responses, we have recently proposed that EP induces these responses by its action on both the peripheral target organs and the CNS, suggesting that there exist two separate mechanisms involved in fever and acute-phase response, one inside and one outside the blood-brain barrier (28, 29). In our recent studies (28, 29), we used the partially purified EP obtained from rabbit leukocytes that were stimulated by lipopolysaccharide of *Salmonella typhosa* endotoxin (32). However, when leukocytes were stimulated by the endotoxin, it is possible that both IL-1 and TNF might have been released (4, 9, 10). Accordingly, it is still unclear whether previous observations of febrile and acute-phase responses induced by partially purified EP resulted mainly from the action of IL-1 or TNF or both.

In the present study, we have further investigated the febrile and acute-phase responses in rabbits after intravenous or intracerebroventricular injections of human recombinant IL-1 and human recombinant TNF. The present results indicate that both IL-1 and TNF have an intrinsic ability to induce fever and acute-phase response. Furthermore, by comparing characteristics of responses induced by intravenous and intraventricular injections of IL-1 with those induced by similar injections of TNF, we suggest that the mechanism by which IL-1

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induces fever and acute-phase response is different from that of TNF.

METHODS

The animals used in this study were male New Zealand White rabbits weighing 3.0–4.0 kg. A total of 21 rabbits was used. Seven animals had been implanted previously with a stainless steel cannula (1.0 mm OD) located in the third ventricle, by standard stereotaxic techniques. This implantation was done under general anesthesia (pentobarbital sodium, 20 mg/kg iv) at least 2 wk before the start of the experiment. IL-1, TNF, or saline was injected directly through the cannula into the third ventricle.

Human recombinant IL-1 α and human recombinant TNF were supplied by the Dainippon Pharmaceutical and had been produced by recombinant strains of *Escherichia coli*. The IL-1 α and TNF were carefully prepared and contained a very low dose of endotoxin, as restrictively confirmed by *Limulus* amoebocyte lysate test (<0.05 pg/ μ g protein). The biological activity of IL-1 α that was assayed by thymocyte costimulation activity was 2×10^7 U/mg protein and that of TNF, which was based on the cytotoxic activity against L-M cells, was 3.15×10^6 U/mg protein. The molecular weight of IL-1 α was 18,000 and that of TNF was 17,000. The isoelectric point (pI) of IL-1 α was 5.3 ± 0.1 and that of TNF was 5.9 ± 0.3 . For injection, the recombinant IL-1 α and TNF were dissolved in sterile saline at a concentration of 100, 10, 1, or 0.1 μ g/ml. These solutions were divided into several vials and stored at -40°C until use. We used each vial within 2 days after thawing and avoided repeat freezing and thawing.

On the day of the experiment, animals were minimally restrained in conventional stocks at an ambient temperature of $21 \pm 1^\circ\text{C}$ between 0830 and 1800 h. To avoid the effect of stress due to restraint, all had been well trained to adapt to the stocks for 6 h every other day, from at least 10 days before the start of experiment. Throughout the experiment, the rectal temperature was measured every minute with a copper-constantan thermocouple. All injections of IL-1 α , TNF, or saline control were performed at 1200 h. Intravenous injections were made into the marginal ear vein through a sterile needle (25 gauge). Intraventricular injections were made through a stainless steel needle (0.6 mm OD) attached to a polyethylene tube, and the volume infused was always 2 μ l. Injection doses in each experimental group are described in the RESULTS, including appropriate control injections.

For measuring the blood cell counts and the plasma concentration of iron, zinc, and copper, ~5 ml of blood were withdrawn through the marginal ear vein. Blood samples were taken three times: 1 h before and 8 and 24 h after injections of IL-1 α , TNF, or saline. Immediately after collecting the blood, the numbers of white blood cells and red blood cells were measured with an automatic cell counter (Coulter, model S plus II). The remaining blood was collected into heparinized polyethylene tubes. It was centrifuged at 2,000 rpm for 15 min at 4°C , and the plasma was collected in the polyethylene tube and stored at -20°C until the measurement of iron, zinc, and

copper concentrations. To determine the plasma iron concentration, 2 ml of HCl (0.1 M) solution containing 10% trichloroacetic acid were added to 0.5 ml of the plasma to denature the proteins. This solution was mixed thoroughly and centrifuged for 10 min at 3,000 rpm. The supernatant was saved for testing. To determine the plasma copper concentration, 1 ml of HCl (0.8 M) and 1 ml of 10% trichloroacetic acid were added to 0.5 ml of plasma and mixed thoroughly. This mixture was centrifuged at 3,000 rpm for 10 min, and the supernatant was saved for testing. To determine the plasma zinc concentration, 2.25 ml of a solution of 6% butanol containing HCl (0.1 M) were added to 0.25 ml of sample and mixed thoroughly. These mixtures were saved for testing. The concentrations of iron, copper, and zinc were measured by a polarized Zeeman atomic absorption spectrophotometer (Hitachi, Z-8000). The absorbance values of samples were read at the wavelength of 248.3 nm for iron, of 324.8 nm for copper, and of 213.8 nm for zinc. The concentrations of iron, copper, and zinc were determined by comparison of absorbance values to a standard curve. Because the plasma concentrations of iron, zinc, or copper were measured by atomic absorption spectrophotometry in the present study, the total iron, zinc, and copper concentrations in the plasma were compared between control and experimental groups.

Data were analyzed for statistical significance by Student's *t* test.

RESULTS

Figure 1 shows the changes in the rectal temperature of rabbits after an intravenous injection of human recombinant interleukin 1 α (IL-1 α) and human recombinant tumor necrosis factor (TNF). In Fig. 1A, the intravenous injection of a smaller dose of IL-1 α (0.5 μ g/kg) produced a monophasic patterned fever and a larger dose (2 μ g/kg) produced a biphasic fever. In both cases the rectal temperature started to increase ~10 min after the injection.

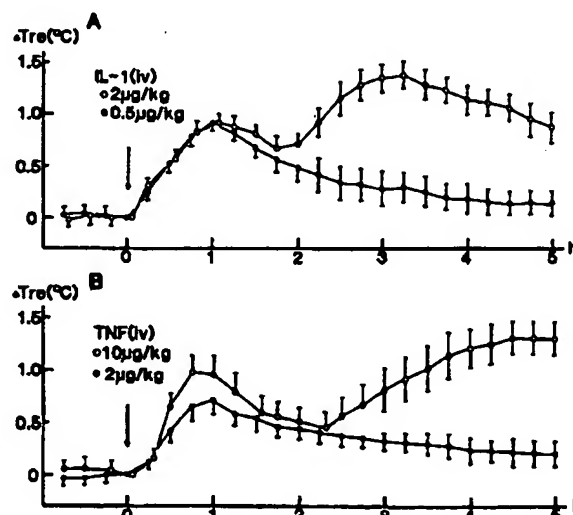


FIG. 1. Mean changes (\pm SE) in rectal temperature (ΔT_{re}) in same group of 7 rabbits after intravenous (iv) injections of human recombinant interleukin 1 α (IL-1 α ; A) and human recombinant tumor necrosis factor (TNF; B).

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tion, and the time to the first peak was 50–70 min. The second peak in the biphasic fever occurred at 160–210 min. Similarly, as shown in Fig. 1B, the intravenous injection of a smaller dose of TNF (2 $\mu\text{g}/\text{kg}$) induced a monophasic fever and a larger dose (10 $\mu\text{g}/\text{kg}$) induced a biphasic fever. The time to the peak in the monophasic fever and the first peak in the biphasic fever was 50–70 min, with the second peak in the biphasic fever occurring at 4–5 h.

The effect of intracerebroventricular injection of IL-1 α and TNF on the rectal temperature is shown in Fig. 2, A and B. About 20 min after intracerebroventricular injection of IL-1 α (A) and TNF (B), the rectal temperature gradually started to rise. The fever induced by intracerebroventricular injection of IL-1 α was markedly prolonged over 4–5 h. In contrast, the febrile pattern induced by intracerebroventricular injection of TNF was monophasic with the peak at 90–120 min. The febrile responses induced by either intracerebroventricular injection of IL-1 α or TNF were found to be dose dependent. The dose-response relationships of the pyrogenic action

IL-1 α and TNF in rabbits are shown in Fig. 3, A and B. The pyrogenicity per gram weight of IL-1 α was apparently greater than that of TNF when injected intravenously (A) or intracerebroventricularly (B).

Figures 4–6 summarize the changes in the plasma levels of iron, zinc, copper, and changes in the circulating leukocyte count 1 h before and 8 and 24 h after intravenous injection of IL-1 α (Fig. 4), TNF (Fig. 5), and after intracerebroventricular injection of IL-1 α or TNF (Fig. 6). In each figure, the changes in each parameter represent the mean \pm SE of the same group of rabbits ($n = 7$). In addition, the values at the respective time are statistically compared between the control group injected with saline and the group injected with IL-1 α or TNF.

In Fig. 4, the intravenous injection of either the smaller dose of IL-1 α (0.5 $\mu\text{g}/\text{kg}$) or the larger dose (2 $\mu\text{g}/\text{kg}$)

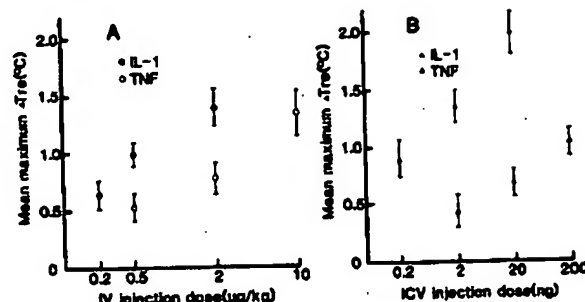


FIG. 3. Mean maximum rise (\pm SE) in rectal temperature (ΔT_{re}) in 7 rabbits after intravenous (IV) or intracerebroventricular (ICV) injections of varying doses of human recombinant interleukin 1 α (IL-1 α) and human recombinant tumor necrosis factor (TNF).

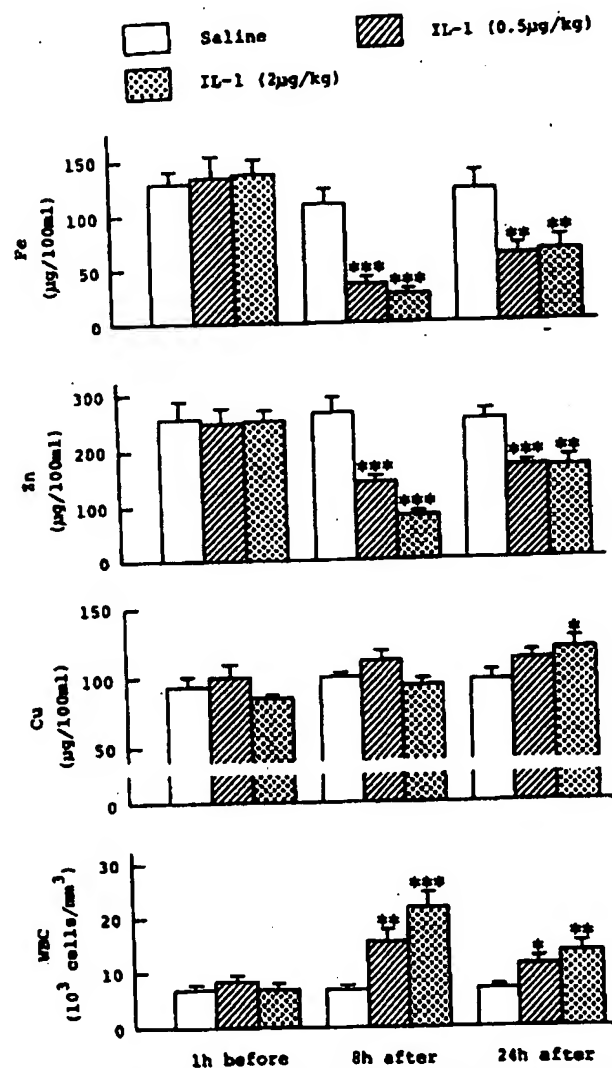


FIG. 4. Changes in plasma concentration of iron (Fe), zinc (Zn), and copper (Cu) and in circulating white blood cell count (WBC) 1 h before and 8 and 24 h after intravenous injections of smaller or larger doses of human recombinant interleukin 1 α (IL-1 α) or saline control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

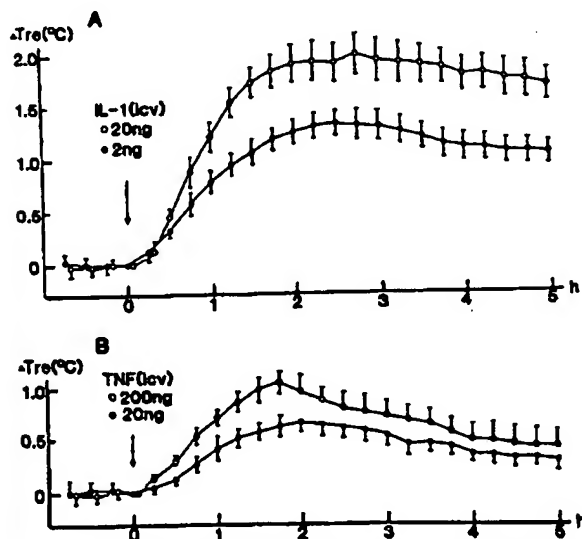


FIG. 2. Mean changes (\pm SE) in rectal temperature (ΔT_{re}) in same group of 7 rabbits after intracerebroventricular (icv) injections of human recombinant interleukin 1 α (IL-1 α ; A) and human recombinant tumor necrosis factor (TNF; B).

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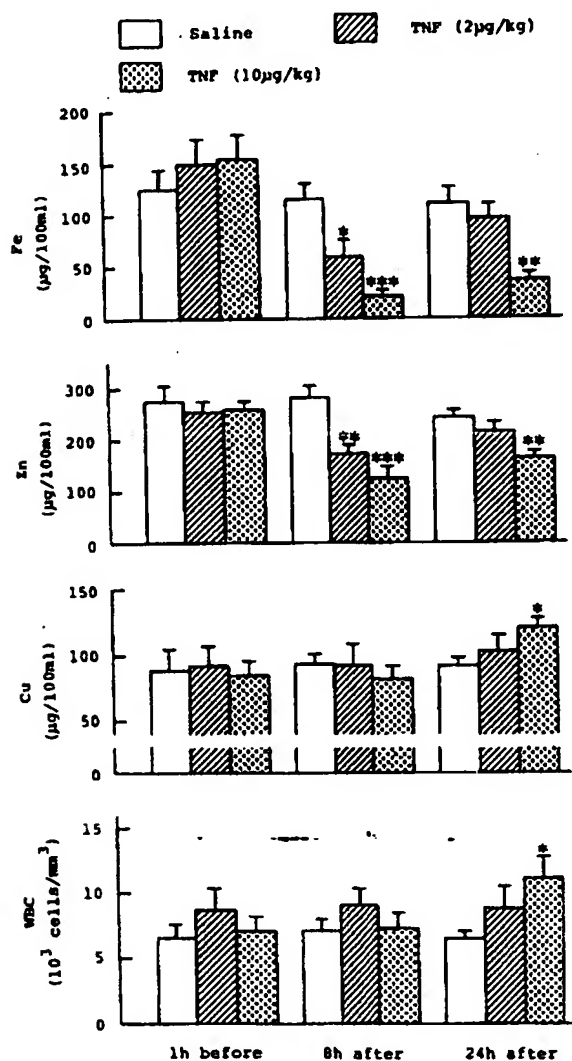


FIG. 5. Changes in plasma concentration of iron (Fe), zinc (Zn), and copper (Cu) and in circulating white blood cell count (WBC) 1 h before and 8 and 24 h after intravenous injections of smaller or larger doses of human recombinant tumor necrosis factor (TNF) or saline control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

significantly decreased the plasma concentration of iron and zinc and increased the circulating leukocyte count 8 and 24 h after injection. Moreover the intravenous injection of the larger dose of IL-1 α increased the plasma copper concentration 24 h after injection.

In Fig. 5, the intravenous injection of the smaller dose of TNF (2 µg/kg) significantly reduced the plasma concentration of iron and zinc 8 h after injection; however, this dose of TNF did not change the plasma concentration of copper and the circulating leukocyte count. The intravenous injection of the larger dose (10 µg/kg) decreased the plasma concentration of iron and zinc 8 and 24 h after injection and increased the plasma copper concentration and the circulating leukocyte count 24 h after injection.

In Fig. 6, the intracerebroventricular injection of IL-1 α (20 ng) decreased the plasma concentration of iron and zinc 8 h after injection, increased the plasma copper

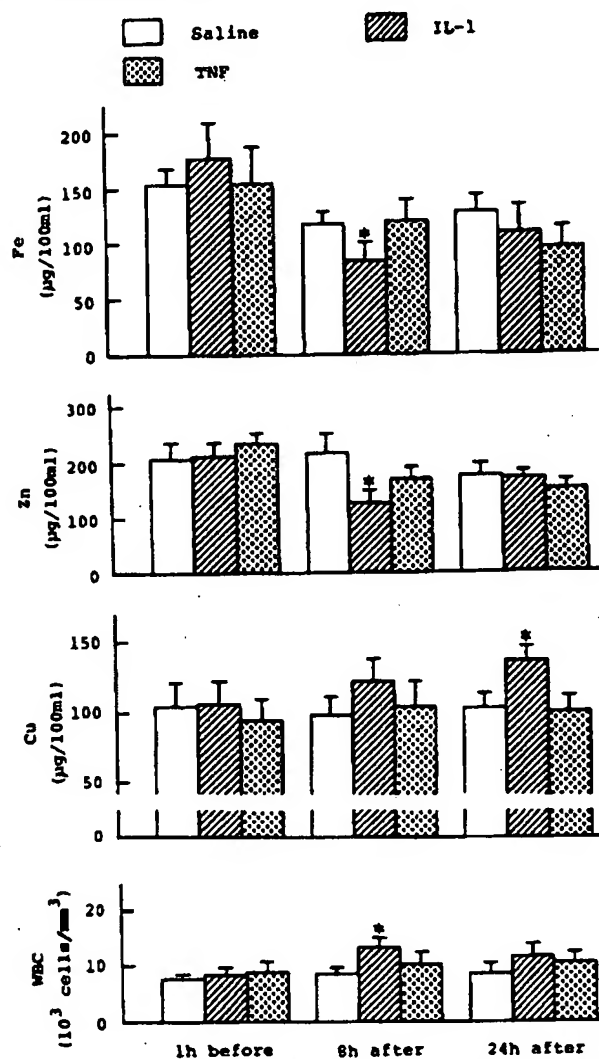


FIG. 6. Changes in plasma concentration of iron (Fe), zinc (Zn), and copper (Cu) and in circulating white blood cell count (WBC) 1 h before and 8 and 24 h after intracerebroventricular injections of human recombinant interleukin 1 α (IL-1 α), human recombinant tumor necrosis factor (TNF), or saline control. * $P < 0.05$.

concentration 24 h after injection, and increased the circulating leukocyte count 8 h after injection. However, the intracerebroventricular injection of TNF (200 ng) did not significantly change any of the parameters.

Furthermore, neither intravenous nor intracerebroventricular injection of IL-1 α or TNF affected the number of red blood cells.

DISCUSSION

In the present study, the effect of human recombinant IL-1 α (hrIL-1 α) and human recombinant TNF (hrTNF) on body temperature and some of the blood parameters related to acute-phase response were investigated by using different doses and different routes of administration. As for the pathogenesis of fever production, we have already shown that small concentrations of partially purified EP injected intravenously produce monophasic fever but that large concentrations produce biphasic

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fever (29, 32). We suggested that there exist two separate mechanisms of fever induction, one inside and one outside the blood-brain barrier (29). The first step in the causation of monophasic fever and in the causation of the first phase of the biphasic fever is that EP acts on structures outside the blood-brain barrier, and subsequently prostaglandins are synthesized and released, which then induce fever. The second step, in which the second phase of the biphasic fever is caused, is that EP acts on structures within the blood-brain barrier to release prostaglandins that cause fever. However, it is still unclear but strongly expected to be determined whether EP in the circulation actually enters the cerebrospinal fluid across the circumventricular organ (6) where the blood-brain barrier is absent or whether an EP-like substance is newly synthesized within the CNS by the action of circulating EP on glial cells, astrocytes (16), and/or cerebral vascular components (36). The present results showed that the intravenous injection of a smaller dose of hrIL-1 α or hrTNF produced monophasic fever and that of a larger dose produced biphasic fever. Accordingly, it is speculated that hrIL-1 α or hrTNF act on structures both outside and inside the blood-brain barrier to induce synthesis and release of prostaglandins and that these act on the CNS to induce fever (10, 12, 14). This speculation seems likely to explain the mechanism of biphasic fever induced by intravenous injection of the larger dose of hrIL-1 α , as an intracerebroventricular injection of hrIL-1 α produced fever similar to the second phase of the biphasic fever. Indeed, the second peak in the biphasic fever induced by intravenous injection of IL-1 α occurred at 160–210 min, whereas the rectal temperature reached the maximum level ~150–180 min after intracerebroventricular injection of hrIL-1 α . However, the second phase induced by the intravenous injection of the larger dose of hrTNF occurred remarkably later than that induced by intracerebroventricular injection of hrIL-1 α . Moreover, intracerebroventricular injection of hrTNF induced monophasic fever with the peak 90–120 min. Hence, it is difficult to accept that the second phase induced by intravenous injection of hrTNF is caused by the action of hrTNF on the CNS. In other words, hrTNF injected intravenously does not seem to act directly on the CNS, although hrTNF by itself is pyrogenic in the CNS. Recently, Dinarello et al. (14) have also reported that the intravenous injection of a large dose of hrTNF induces a biphasic fever in rabbits. They concluded that the second phase induced by hrTNF is caused by IL-1 newly produced by TNF, because the plasma IL-1 concentration increased during the second phase. As will be described later, judging from the present results obtained from acute-phase response, their conclusion is supported.

Furthermore, the pyrogenicity per gram of hrIL-1 α was greater than that of hrTNF when injected intravenously or intracerebroventricularly. However, according to the result by Dinarello et al. (14), the ability of hrIL-1 and hrTNF to produce prostaglandin E $_2$ in incubated slice preparations of rabbit hypothalamus was almost the same. Therefore it remains to be understood why the pyrogenicity of hrIL-1 α injected into the third ventricle

was greater than that of hrTNF.

The intravenous injection of either smaller or larger doses of hrIL-1 α decreased the plasma concentration of iron and zinc and increased the circulating leukocyte count 8 and 24 h after injection. Moreover, the larger dose increased the plasma copper concentration 24 h after injection. In contrast, the intracerebroventricular injection of hrIL-1 α decreased the plasma concentration of iron and zinc 8 h after injection, increased the plasma copper concentration 24 h after injection, and increased the circulating leukocyte count 8 h after injection. These results indicate that, as we recently suggested (28), hrIL-1 induces the acute-phase response directly through its action on several peripheral target organs [muscle, bone marrow and, many others (19)] as well as indirectly via neural output originating from its action on the CNS (7, 35). Furthermore, we now must take into account a previous demonstration that the effect of IL-1 on hepatic synthesis of acute-phase proteins is mediated by hepatocyte-stimulating factor (18), which was recently renamed as interleukin 6 (17, 33). Considering that the intravenous injection of a smaller dose did not affect the level of the plasma copper concentration but the intravenous injection of the larger dose and the intracerebroventricular injection increased it 24 h after injection, it seems that the plasma copper concentration is mainly controlled by the central action of IL-1. In addition, it is apparent that the central effect of IL-1 on the acute-phase response is not mediated by arachidonic acid metabolites, which are induced by IL-1, because intracerebroventricular injection of arachidonic acid or prostaglandin E $_2$ does not induce the acute-phase response (31). Based on the results of changes in the plasma copper concentration, it is further suggested that the monophasic fever induced by a smaller dose of intravenous IL-1 is caused by prostaglandins, which IL-1 does not induce by acting on structures inside the blood-brain barrier but by acting on structures outside the blood-brain barrier.

In a comparison of our recent results (28) using partially purified EP with the present results, remarkable differences are noticed in that the intracerebroventricular injection of EP decreased the plasma zinc concentration 24 h after injection, whereas that of hrIL-1 did so 8 h after injection. There are two distinct forms of IL-1 with disparate amino acid sequences (15, 27); IL-1 α (pI 5) and IL-1 β (pI 7), and it has been reported that IL-1 β is the predominant type produced by human monocytes (11). Therefore it is at present unknown if functional differences exist in the action of IL-1 between the two types of IL-1s and, moreover, whether structural and functional differences exist between human and rabbit IL-1.

The intravenous injection of a smaller dose of hrTNF decreased the plasma concentration of iron and zinc 8 h after injection. Moreover, a larger dose decreased the plasma concentration of iron and zinc 8 and 24 h after injection and increased the plasma copper concentration and the circulating leukocyte count 24 h after injection. However, the intracerebroventricular injection of hrTNF affected neither the plasma concentration of iron, zinc,

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and copper nor the circulating leukocyte count. This indicates that hrTNF induces acute-phase response only by its action on the peripheral target organs outside the blood-brain barrier. Therefore, in light of the fact that Dinarello et al. (14) suggested that the second phase of the biphasic fever induced by intravenous injection of TNF is caused by IL-1 newly produced by TNF, it is likely that, in the present results, changes in the plasma concentration of iron, zinc, and copper and increases in the circulating leukocyte count 24 h after intravenous injection of the larger dose of hrTNF, which induced the biphasic fever, were mainly caused by peripheral and central actions of IL-1, which is peripherally produced by hrTNF. However, hrTNF does not seem to induce IL-1 synthesis inside the blood-brain barrier, since the intracerebroventricular injection of TNF did not induce acute-phase response. Furthermore, it is inferred that the concentration of IL-1 produced by the larger dose of hrTNF was considerably high, because increases in the plasma copper concentration occurred 24 h after injection, which was observed elsewhere in only the case of intravenous injection of a larger dose of hrIL-1.

In the present study, we reported that two kinds of monokines, hrIL-1 α and hrTNF, are intrinsically pyrogenic and induce acute-phase response, although different mechanisms seem to exist between hrIL-1 and hrTNF. Thus it is apparent that fever and acute-phase response are induced by several kinds of cytokines that are IL-1, TNF, and interferon- γ (30), indicating the possibility that these responses are induced by products not only from monocytes but also from other kinds of leukocytes. Moreover, because of the close interactions of the immunoregulatory networks among various kinds of cells, it is possible that several kinds of cytokines may contribute mutually and simultaneously to the induction of naturally occurring fever and acute-phase response under such pathological conditions as infections (10, 11, 25). In fact, it has been suggested that interferon- γ (1, 30) and TNF (26) induce the synthesis and release of IL-1 in addition to and despite their intrinsic pyrogenicity. Furthermore, considering that several kinds of cytokines induce fever and acute-phase response, it is inferred that fever and acute-phase response enhance immunologic reactions for host defense (23).

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◆ TRENDS IN MOLECULAR MEDICINE ◆

Matrix metalloproteinases and their inhibitors in tumour growth and invasion

Veli-Matti Kähäri¹ and Ulpu Saarialho-Kere²

Controlled degradation of the extracellular matrix (ECM) is crucial for the growth, invasive capacity, metastasis and angiogenesis of tumours. Matrix metalloproteinases (MMPs), a family of zinc-dependent neutral endopeptidases that are collectively capable of degrading essentially all ECM components, apparently play an important role in all of these aspects of tumour development. In addition, there is recent evidence that MMPs are also important for tumour cell survival. At present, therapeutic intervention on tumour growth and invasion based on the inhibition of MMP activity is under intensive investigation, and several MMP inhibitors are already being used on malignant tumours of various organs in clinical trials. In this review we discuss the role of MMPs and their inhibitors in tumour invasion as a basis for prognostic purposes and for targeted therapeutic intervention in cancer.

Key words: cancer; invasion; matrix metalloproteinase.

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Introduction

Controlled breakdown of the extracellular matrix (ECM) is an essential feature of connective tissue remodelling in such physiological conditions as developmental tissue morphogenesis, tissue repair and angiogenesis. On the other hand, excessive breakdown of the components of connective tissue apparently plays a pathogenetic role, eg in rheumatoid arthritis, osteoarthritis and periodontitis. Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases collectively capable of degrading essentially all components of the ECM. In addition to the pathological situations mentioned above, MMPs have been implicated eg in dermal photoageing,

atherosclerosis, myocardial infarction, multiple sclerosis, as well as in tumour cell invasion and metastasis (1–3).

Matrix metalloproteinases

At present, the human MMP gene family contains at least 16 structurally related members, which can be divided into subgroups of collagenases, gelatinases, stromelysins and stromelysin-like MMPs, membrane type-MMPs (MT-MMPs), and novel MMPs according to their primary structure and substrate specificity (Fig 1). In general, MMPs contain a signal peptide, a propeptide, a catalytic domain with the highly conserved zinc-binding site, and a haemopexin-like domain linked to the catalytic domain by a hinge region (Fig 1). In addition, gelatinase-A (MMP-2) and gelatinase-B (MMP-9) contain fibronectin type II inserts within the catalytic domain, and MT-MMPs contain a transmembrane domain at the C-terminal end of the haemopexin-like domain (Fig 1). The haemopexin domain is absent in the smallest MMP, matrilysin (MMP-7). The substrate specificity of distinct MMPs has been determined on the basis of their ability to degrade different components of ECM

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Abbreviations and acronyms

ECM	extracellular matrix
EMMPRIN	extracellular matrix metalloproteinase inducer
ERK	extracellular signal-regulated kinase
IL	interleukin
JNK	Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MMPI	MMP inhibitor
MT-MMP	membrane-type MMP
PKC	protein kinase C
SAPK	stress-activated protein kinase
SCC	squamous cell carcinoma
TACE	TNF- α -converting enzyme
TGF- β	transforming growth factor- β
TIMP	tissue inhibitor of MMPs
TK	tyrosine kinase
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor

in vitro (1–3). However, direct evidence for the proteolytic activity of MMPs *in vivo* is still limited.

Collagenases

Collagenase-1 (MMP-1), collagenase-2, (MMP-8) and collagenase-3 (MMP-13) are the principal secreted neutral proteinases capable of initiating degradation of native fibrillar collagens of types I, II, III and V. They all cleave fibrillar collagens at a specific site resulting in the generation of N-terminal 3/4 and C-terminal 1/4 fragments, which then rapidly denature at body temperature and are further degraded by other MMPs, eg gelatinases (1–3). In addition, human MMP-13 cleaves type I collagen in the N-terminal nonhelical telopeptide (4). MMP-13 has an exceptionally wide substrate specificity and limited expression compared with other MMPs. MMP-13 cleaves fibrillar collagens with preference to type II collagen over type I and III collagens and displays over 40 times stronger gelatinase activity than MMP-1 and MMP-8 (5–7). MMP-13 also degrades type IV, X and XIV collagens as well as tenascin, fibronectin and the aggrecan core protein (8, 9). MMP-1 is expressed by various types of cells in culture and *in vivo*, whereas MMP-8 is produced by neutrophils and chondrocytes (1–3). Apparently, because of its ability to degrade a wide range of ECM components, the physiological expression of MMP-13 is limited to situations in which rapid and effective remodelling of collagenous ECM is required, ie fetal bone development and postnatal bone remodelling (10, 11). On the other hand, MMP-13 is expressed at sites of excessive degradation of collagenous ECM in osteoarthritic cartilage (7, 11), rheumatoid synovium (11, 12), chronic cutaneous ulcers (13), intestinal ulcerations

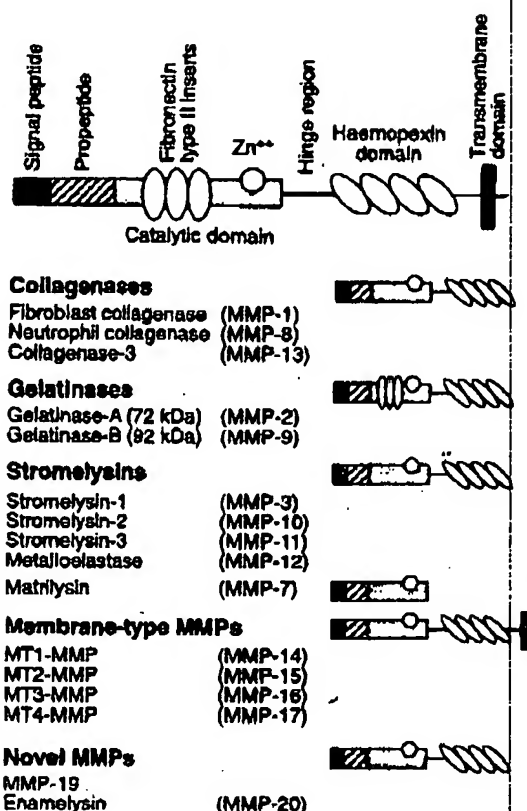


Figure 1. Structure of human matrix metalloproteinases.

(14) and periodontitis (15), as well as in malignant tumours, ie breast carcinomas (5, 16, 17), squamous cell carcinomas (SCCs) of the head and neck and the vulva (18–20), cutaneous basal cell carcinomas (19) and chondrosarcomas (21).

Gelatinases

Gelatinase-A (a 72-kDa (kilodalton) gelatinase, MMP-2) and gelatinase-B (a 92-kDa gelatinase, MMP-9) were initially called 72-kDa and 92-kDa type IV collagenases, respectively. MMP-2 is expressed by various types of cells, especially fibroblasts, whereas the expression of MMP-9 is more restricted: it is produced by epithelial cells, eg keratinocytes, and stored in the secretory granules of neutrophils and eosinophils (22). MMP-2 and MMP-9 are thought to play an important role in the final degradation of fibrillar collagens after they have first been cleaved by collagenases and denatured. MMP-2 can also cleave native type I collagen to N-terminal 3/4 and C-terminal 1/4 fragments identical to those generated by collagenases (23). In addition, MMP-9 has been shown to cleave type I, II and V collagens in the N-terminal nonhelical telopeptide (24). Thus, it is possible that MMP-2 and

MMP-9 also play a role in the remodelling of collagenous ECM under certain conditions.

Stromelysins and stromelysin-like MMPs

Stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) are closely related with respect to structure and substrate specificity (1–3). MMP-3 and MMP-10 are both expressed by epithelial cells, such as keratinocytes and fibroblasts, as well as by various types of malignant cells, and they are able to degrade a wide range of substrates, eg fibronectin, type IV, V, IX and X collagens, elastin, laminins, gelatin and proteoglycan core proteins.

Stromelysin-3 (MMP-11), matrilysin (MMP-7) and macrophage metalloelastase (MMP-12) are often included in this subgroup although they are structurally less closely related to MMP-3 and MMP-10. MMP-11 degrades serine proteinase inhibitors, but it has not been shown to degrade any ECM components. MMP-11 is expressed by fibroblastic cells. Matrilysin (MMP-7), the smallest member of the MMP gene family, lacks the haemopexin-like domain in the C-terminus. MMP-7 has a wide substrate specificity: it is able to degrade fibronectin, laminin, nidogen, type IV collagen and proteoglycan core proteins. Macrophage metalloelastase (MMP-12) degrades elastin, type IV collagen, fibronectin, vitronectin and laminin. It is expressed by macrophages and stromal cells at sites of rapid matrix turnover during murine fetal development (3) and in granulomatous diseases of the intestine and skin (14, 25).

Membrane-type MMPs

The MT-MMP subgroup consists of four members, all of which contain a transmembrane sequence within the carboxy terminal haemopexin domain. MT1-MMP (MMP-14), MT2-MMP (MMP-15) and MT3-MMP (MMP-16) have been shown to activate latent MMP-2 proteolytically (26, 27), and MT1-MMP and MT2-MMP have also been shown to activate latent MMP-13 (28). MT1-MMP degrades the native type I collagen, gelatin, fibronectin, laminin-1 and the cartilage proteoglycan core protein (29). MT2-MMP has been shown to degrade laminin, fibronectin and tenascin (30), and MT3-MMP has been shown to degrade gelatin, type III collagen and fibronectin (31, 32). The substrate specificity of MT4-MMP is not known.

New MMPs

Two recently cloned novel human members of the MMP family, MMP-19 (33) initially named MMP-18 (34), and enamelysin (MMP-20) (35), cannot be classified to any of the MMP subgroups on the basis

of their structure and substrate specificity. MMP-19 is expressed in various tissues, and its ability to degrade native ECM components is not known. MMP-20 is expressed during tooth development and has been shown to degrade amelogenin.

Regulation of MMP expression and activity

Transcriptional regulation

In general, MMPs are not constitutively expressed by cells *in vivo*, but their expression is rapidly induced in response to exogenous signals, eg cytokines or growth factors and altered cell-matrix and cell-cell interactions (1–3). As exceptions to this rule, collagenase-2 (MMP-8) and the 92-kDa gelatinase (MMP-9) are stored in secretory granules of neutrophils and eosinophils (22), and matrilysin (MMP-7) is stored in the secretory epithelial cells of exocrine glands of, for example, the skin, gastrointestinal tract and airways (36, 37). The expression of MMPs is primarily regulated at the level of transcription, and their proteolytic activity is regulated by zymogen activation and inhibition of activity. The basal expression of several MMPs (1, 3, 7, 9, 10, 12 and 13) in cultured cells is low, and their transcription is induced by a variety of extracellular stimuli, such as cytokines and growth factors (1, 2). The 5'-flanking regulatory regions of these inducible MMPs contain an AP-1 *cis*-regulatory element in the proximal promoter approximately at position -70 with respect to the transcription initiation site (38).

The extracellular signals mentioned above activate the nuclear AP-1 transcription factor complex composed of the members of Jun and Fos proteins, which bind to the AP-1 *cis*-element and activate transcription of the corresponding MMP gene. The expression of the components of the classical AP-1 dimer, c-Jun and c-Fos, are induced as a result of the activation of three distinct classes of mitogen-activated protein kinases (MAPKs), ie the extracellular signal-regulated kinase (ERK), stress-activated protein kinase/Jun N-terminal kinases (SAPK/JNKs) and p38. In general, the ERK1,2 cascade is activated by mitogenic signals, resulting in the phosphorylation of various substrates, including Elk-1 by ERKs, and in the subsequent activation of c-Fos transcription. The SAPK/JNKs and p38 are activated by cytokines (tumour necrosis factor (TNF), interleukin (IL)-1) and stress stimuli, such as UV light. The activation results in the phosphorylation of c-Jun and ATF-2 by JNKs, and that of ATF-2 by p38, which in turn results in the induction of c-Jun transcription (39, 40).

Another important *cis*-element, the PEA3 site, is also present in the promoter regions of AP-1-responsive MMPs mentioned above, but its location and number are more variable than those of the AP-1

cis-element. The PEA3 element binds members of the ETS family of transcription factors, and it has been shown to cooperate with the AP-1 motif for maximal activation of MMP-1, MMP-3 and MMP-9 promoters (41–43). Expression of ETS-1 has been demonstrated in stromal fibroblasts adjacent to invading tumour cells and in endothelial cells during tumour vascularization (44–46).

Activation of latent MMPs

Most MMPs are secreted as latent precursors (zymogens), which are proteolytically activated in the extracellular space with the exception of stromelysin-3 (MMP-11) and MT1-MMP, which are activated prior to their secretion by Golgi-associated furin-like proteases (47). The proMMPs are retained in latent form by a 'cysteine switch' formed by the interaction of a conserved cysteine in the propeptide with the catalytic zinc blocking the access of the catalytic site to substrate (48). Activation of the latent MMP by chaotropic agents (eg organomercurials), or partial proteolytic cleavage of the propeptide, dissociates the covalent bond between the cysteine and the catalytic zinc and exposes the catalytic site. For example, MMP-1 is activated by several proteases, eg plasmin, MMP-3 and MMP-10, and it can activate latent MMP-2. Latent MMP-13, in turn, is activated by plasmin, MT1-MMP, MMP-3, MMP-10 and MMP-2, and it can activate MMP-2 and MMP-9 (28, 49). Thus, the ability of MMPs to activate each other creates a complex network of proteases in the pericellular space. Furthermore, activation of latent MMP-2 by membrane-anchored MT1-MMP and MT2-MMP (26, 27), and interaction of MMP-2 with the $\alpha\beta 3$ integrin on cell membrane (50) are examples of mechanisms for directing proteolytic activity to focal areas in the pericellular space.

Inhibition of MMP activity

The proteolytic activity of MMPs is inhibited specifically by tissue inhibitors of metalloproteinases (TIMPs) as well as by nonspecific inhibitors, including the $\alpha 2$ -macroglobulin and the $\alpha 1$ -antiprotease. Serum protease inhibitors, eg $\alpha 2$ -macroglobulin, are abundant in the extracellular space everywhere in the human body, and they apparently play an important role in controlling the overall proteolytic activity in tissues.

Tissue inhibitors of metalloproteinases

The activity of MMPs is specifically inhibited by TIMPs, which bind to the highly conserved zinc-binding site of active MMPs at molar equivalence. Currently, the TIMP gene family consists of four structurally related members, TIMPs 1, 2, 3 and 4,

which show a 30–40% identity at the amino acid level and possess 12 conserved cysteine residues required for the formation of six loops (51). TIMPs 1, 2 and 4 are secreted in soluble form, whereas TIMP-3 is sequestered to the ECM (52). TIMP-1 and TIMP-2 inhibit the activity of most MMPs with the exception of MT1-MMP, the activity of which is not inhibited by TIMP-1. TIMP-2 is a 10 times more effective inhibitor of gelatinases, whereas TIMP-1 is about twice more effective against MMP-1 than against other MMPs. Furthermore, TIMP-1 and TIMP-2 form complexes with latent MMP-9 and MMP-2, respectively (51). TIMP-3 inhibits the activity of MMPs 1, 2, 3, 9 and 13 (28) and unlike other TIMPs it inhibits the activity of the tumour necrosis factor (TNF)- α -converting enzyme (TACE) (53), suggesting that it plays a role in the modulation of inflammatory response by inhibiting shedding and activation of TNF- α . TIMP-4 inhibits the activity of MMP-2 and MMP-7 somewhat more potently than that of MMPs 1, 3 and 9 (54).

TIMPs are produced by a variety of cell types, such as fibroblasts, keratinocytes, endothelial cells and osteoblasts. Expression of TIMP-1 is regulated at the level of transcription by various growth factors, hormones and cytokines, such as transforming growth factor- β (TGF- β), IL-1 and IL-6, retinoic acid, TNF- α , epidermal growth factor and glucocorticoids, whereas TIMP-2 is largely expressed constitutively by cultured cells. TIMP-3 expression is up-regulated by mitogens, phorbol esters and TGF- β , and inhibited by TNF- α (52, 55, 56). Furthermore, it is regulated in a cell cycle-dependent manner in several types of cells (57). The expression of TIMP-4 is restricted: it is abundantly expressed in the adult human heart and, at very low levels, in the kidney, placenta, colon and testis (58).

TIMPs have biological effects that extend beyond their role as inhibitors of MMP activity. They induce changes in cell morphology, stimulate growth of several cell types and are involved in steroidogenesis and germ cell development of both sexes (51). In addition, TIMP-1 and TIMP-3 are antiangiogenic (59–61). Interestingly, TIMP-2 is also involved in the activation of MMP-2 as it binds to latent MMP-2 and MT1-MMP at cell surface, resulting in proteolytic activation of the latent MMP-2 by adjacent MT1-MMP (26, 27). In addition, over-expression of TIMP-1 has been shown to stimulate the production of vascular endothelial growth factor (VEGF), type IV collagen and laminin by breast carcinoma cells in culture (62, 63).

MMPs and TIMPs in tumour invasion

Role of MMPs in tumour invasion

Tumour invasion is a multistep process in which cellular motility is coupled to proteolysis and involves

interactions with the ECM. During invasion, malignantly transformed cells detach from the primary tumour, migrate and cross structural barriers, including basement membranes and surrounding stromal ECM consisting mainly of fibrillar collagens. In addition, proteolytic degradation of stromal ECM is considered essential in tumour-induced angiogenesis.

However, the proposed role of MMPs in tumour invasion is, at present, mainly based on the observations of abundant expression of distinct MMPs in invasive primary tumours or in their metastases (reviewed in (64–66)); whereas evidence for the actual activity of distinct MMPs in tumour tissues *in vivo* is limited. As the expression of most MMPs is low or undetectable in normal tissues, activation of their expression in malignant tumours *in vivo* has been taken to indicate that they are, in fact, produced and activated *in situ*. This notion is supported by observations that expression of certain MMPs is associated with the invasion capacity of certain tumours. For example, the level of MMP-1 expression correlates with a poor prognosis of colorectal and oesophageal cancer (67, 68), and the expression of MMP-2 and MMP-3 is closely related to lymph node metastases and vascular invasion in SCCs of the oesophagus (69). Similarly, abundant expression of MMP-13 in SCCs of the head and neck and vulva is associated with their metastatic capacity (18, 20). MMP-11 expression is also associated with increased local invasiveness of SCCs of the head and neck (70). Increased expression of gelatinases and collagenases correlates with the invasiveness of malignant melanomas (71, 72).

In vivo, MMP-2 is observed early in melanocytic tumour progression, in junctional nests of benign nevi, with the level of MMP-2 expression increasing with advancing architectural disorganization and atypia, suggesting a role for MMP-2 in melanoma invasion and metastasis (71). In the light of the activation mechanism of MMP-2, it is not surprising that the expression of MMP-2, TIMP-2 and MT1-MMP correlates with poor prognosis of bladder cancer (73). Similarly, expression of MMP-2 and MT1-MMP correlates with malignant progression in gliomas (74). These observations show that it may be more informative to compare the invasion capacity of malignant tumours with the expression of several MMPs and their activators.

Additional direct evidence for the role of MMP activity in invasion and growth of tumours has been recently provided by knock-out mice for distinct MMPs. MMP-7 knock-out mice showed reduction in intestinal tumourigenesis (75) and MMP-2-deficient mice show reduced angiogenesis and tumour progression (76). MMP-11 knock-out mice show reduced tumourigenesis in response to chemical mutagenesis (77).

In most tumours, stromal fibroblasts are the

primary source of MMPs. In many tumours, stromal cells also actively express ECM components, eg type I collagen, indicating that the surrounding stromal compartment of malignant tumours is undergoing extensive tissue remodelling (78, 79). Infiltration of inflammatory cells is also a prominent feature of many malignant tumours, and they can also be a source of certain MMPs in the peritumoural environment. For example, in SCC tumour cells, stromal fibroblasts and inflammatory cells all express a distinct set of MMPs, which can at least in part complement the proteolytic capacity of each other (20). In addition to MMPs, inflammatory cells produce cytokines, which may enhance expression of MMPs by tumour and stromal cells. Furthermore, tumour cells produce proteins, such as EMMPRIN (extracellular matrix metalloproteinase inducer), which enhances the production of MMPs by fibroblasts (80, 81).

It is likely that distinct MMPs form a network, in which a single MMP is crucial for the cleavage of certain native or partially degraded matrix components and for the activation of other latent MMPs. It is therefore possible that the inhibition of the expression or activity of only one MMP can potentially reduce peritumoural proteolytic activity and tumour invasion. It is also possible that different MMPs play distinct roles at different stages of tumour development. The stromal reaction is often regarded as a host response to tumour (16). In this context, it is interesting that MMPs 3, 7, 9 and 12 have been shown to generate angiostatin from plasminogen, indicating that their expression in peritumoural area may serve to limit tumour-induced angiogenesis (82–84). It should be noted, that all MMPs, the expression of which has been documented in malignant tumours, can also be expressed by non-neoplastic cells. MMP-13, MMP-7 and MT1-MMP can be regarded as transformation-specific MMPs, at least in keratinocytes, as they are not expressed by normal keratinocytes, or in premalignant lesions of the skin (actinic keratoses and Bowen's disease). However, they are readily expressed by malignantly transformed keratinocytes in SCCs, indicating that their expression serves as a marker for transformation and invasion capacity of SCC cells (18–20, 85).

Role of TIMPs in tumour invasion

The ability of TIMPs 1, 2, 3 and 4 to inhibit tumour growth has been shown by over-expressing them in various human and rodent cell lines (86–89). In transgenic mice, TIMP-1 over-expression inhibited primary growth of T-cell lymphoma. However, metastatic colonization of the liver was not hindered by elevated TIMP levels (90), suggesting that not only the tumour cells but also the host stroma are integral to cancer progression. Effects of tumour vs host

expression of TIMP-1 are further illustrated in recent studies on genetically manipulated mice, varying in the expression of TIMP-1, in which lung colonization was influenced by the TIMP-1 genotype of the tumour but not by that of the host (91).

In malignant tumours *in vivo*, MMPs often colocalize with their specific inhibitors. Both TIMP-1 and MMP-2 were expressed in nearly all the head and neck carcinomas examined (92). TIMP-1 and TIMP-2 have often been detected in cancer and endothelial cells (72) and very typically together with MMPs in stromal cells adjacent to malignant tumours (93, 94). In SCCs the presence of TIMP-1 and TIMP-2 correlated with less aggressive behaviour (94). TIMP-2 expression has been detected, for example, in the stroma of colorectal carcinomas, and it has been suggested that abundant expression of TIMP-2 in the stroma would correlate with longer survival time (95). Similarly, higher levels of TIMP-2 were detected in basal cell carcinomas than in SCCs, indicating an inverse correlation between TIMP-2 expression and invasive capacity of these cutaneous tumours (96). Stromal cells of colon and mammary carcinomas express TIMP-3 (55). It is not expressed in benign skin tumours, but infiltrative basal cell carcinomas express TIMP-3 in the malignant cells at the margins of tumour islands (55), whereas both TIMP-1 and TIMP-2 are detected only in the stromal cells surrounding the tumours (96). In well-differentiated cutaneous and oral SCCs, stromal cells adjacent to the tumour express TIMP-3 and TIMP-1 (55, 97). In melanomas, TIMP-1 and TIMP-3 expression is increased in tumour and stromal cells in invasive melanomas (72). Expression of TIMP-4 in malignant tumours *in vivo* has not been documented.

In some malignant tumours, a correlation has been detected between the invasiveness and prognosis of the tumour and the ratio of MMPs and TIMP-1 and TIMP-2 (98). This correlation could be used to determine whether patients with advanced cancer need adjuvant chemotherapy after complete resection (99). However, high TIMP levels in various types of malignant tumours also correlate with more aggressive behaviour of the tumour. An association between a poor prognosis and high levels of expression of TIMP-1 in colorectal (100) and non-small-cell lung cancers (101) and that of TIMP-2 in bladder cancer has been reported (102). Increased expression of TIMPs 1, 2 or 3 in highly malignant tumours of the gastrointestinal tract (103, 104), breast (105), lung (106), head and neck (93), and the skin (72) has been demonstrated. These reports suggest that TIMPs have a dual effect on tumour growth. In addition to suppressing proteolysis and neovascularization they may promote tumour cell proliferation at some stage of tumour progression (51). Whether abundant expression of TIMPs by stromal cells indicates a stronger host

response against a more invasive tumour or whether TIMPs are needed for tumour growth or invasion cannot be answered conclusively at present.

Prevention of tumour growth and invasion by inhibiting MMP activity

Based on the observations discussed above it has been suggested that inhibition of MMP activity could markedly inhibit invasion and metastasis of neoplastic cells and effectively suppress growth of the primary tumour and metastases by inhibiting angiogenesis (107). In general, MMP expression or activity can be inhibited at several steps in the synthetic pathway of MMPs (Fig 2).

Inhibition of MMP transcription

An effective way of inhibiting the activity of MMPs may be to target their transcriptional activity either by blocking signalling pathways regulating MMP transcription or by inhibiting transcription factors responsible for activating transcription of MMP genes. It has been shown that inhibition of signalling pathways involved in AP-1 activation, ie the MAPKs (ERK1,2, SAPK/JNK or p38) can markedly inhibit the expression of MMPs and the invasion of malignant cells *in vitro*. Inhibition of the ERK1,2 pathway (Raf/MEK1,2/ERK1,2) by a specific inhibitor of MEK1,2 activation (PD 98059) potently inhibits urokinase expression in SCC cells (108) and MMP-1 expression in fibroblasts (109). In addition, inhibition of p38 activity by a specific inhibitor, SB 203580, abrogates activation of MMP-1 and MMP-13 expression in

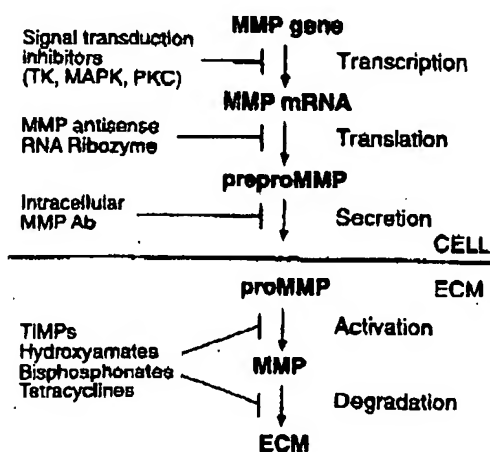


Figure 2. Inhibition of matrix metalloproteinase expression and activity at distinct levels of expression. For definitions, please refer to the list of abbreviations.

fibroblasts (109–111) as well as MMP-9 expression and invasion of SCC cells (112). However, the efficacy of MAPK inhibitors in the *in vivo* invasion of malignant cells has not been demonstrated.

Expression of dominant negative c-Jun has been shown to inhibit the induction of MMPs 1, 2, 3, 10 and 14 as well as the *in vitro* invasion of immortalized keratinocytes (113). Similarly, stable expression of dominant-negative cyclic AMP response element-binding protein (CREB) inhibits the expression of MMP-2 and the invasion of malignant melanoma cells *in vivo* (114). A disadvantage in this approach is that other transcription factors, which may play a role in the activation of MMP expression, are not necessarily affected. Furthermore, utilization of dominant-negative transcription factors as a form of cancer therapy would require an effective method of gene delivery *in vivo*. Retinoids as inhibitors of AP-1 activity could also be used to prevent MMP gene expression in selected human carcinomas (66).

Inhibition of MMP translation

Another option for inhibiting expression of specific MMPs is to use antisense RNAs. Antisense oligonucleotides for MMP-7 have been shown to inhibit invasion of colon carcinoma cells *in vitro* and *in vivo* (115). This approach may benefit from the addition of a hammerhead ribozyme loop to the antisense RNA to allow effective degradation of specific messenger RNA molecules. An MMP-9 antisense-ribozyme expression construct was shown to inhibit expression of MMP-9 and the invasion of rat osteosarcoma cells (116). Clearly, this approach also requires an effective method of gene transfer to the cells *in vivo*.

Inhibition of MMP activity

At present, inhibition of MMP activity in the extracellular space has been the most extensively studied approach to inhibit invasion of neoplastic cells. The ability of TIMPs to potently and specifically inhibit MMP activity has drawn attention to them. Several experimental studies have shown that over-expression of TIMPs 1, 2, 3 and 4 can inhibit invasion of malignant cells *in vitro* and *in vivo* (91). In addition, over-expression of TIMP-3 induces apoptosis in various types of malignant cells, suggesting that MMP activity plays an important role in the survival of tumour cells (88, 117, 118). However, TIMP-based cancer therapy would clearly require an effective method of gene delivery, such as recombinant adenoviruses (88, 118).

Synthetic MMP inhibitors (MMPIs), such as hydroxamate inhibitors, are small ($M_r < 600$) peptide analogues of fibrillar collagens, which specifically interact with the zinc in the catalytic site of MMPs

and inhibit their activity. At present, several MMPIs aimed at treating invasive malignant tumours, such as gastric, pancreatic and ovarian cancers, are in clinical trials (Table 1) and might represent a new approach to cancer treatment, complementary to traditional cytotoxic drugs. Synthetic MMPIs may inhibit tumour growth either by enhancing the development of the fibrotic capsule around the tumour, and thereby preventing tumour invasion, or by inhibiting tumour-induced angiogenesis. The principal side-effect of these drugs has been musculoskeletal pain apparently resulting from the inhibition of MMP activity involved in normal turnover of connective tissue in tendons and joints (107).

Broad-spectrum hydroxamate MMPIs, batimastat (BB-94) and marimastat (BB-2516), were the first MMPIs to enter clinical trials in the treatment of malignant tumours. Batimastat is well tolerated, but its utility is limited by poor water solubility, which requires intraperitoneal administration (119). Marimastat is water soluble and can be administered orally. At present, marimastat is used in phase II and III clinical trials in North America and Europe in the treatment of several malignant tumours. Selective targeting of certain MMPs instead of using broad-spectrum inhibitors has been suggested to be preferable because of the fact that certain MMPs (MMPs 3, 7, 9 and 12) can inhibit angiogenesis by generating angiostatin from plasminogen, and thereby suppress tumour growth. Specific MMPIs targeted against collagenases or gelatinases have been developed, but the complex effects of MMP inhibitors are demonstrated by findings that MMPIs with similar inhibition profile *in vitro* can have entirely opposite effects on tumour growth and metastasis *in vivo* (107).

Tetracyclines have the ability to block host-derived MMPs by a mechanism independent of their antimicrobial effect (120). Recent observations indicate

Table 1. Synthetic matrix metalloproteinase inhibitors in clinical trials of cancer.

Compound	Company	Phase of clinical trial
BB-94, Batimastat	British Biotech	Phase II/cancelled
BB-2516, Marimastat	British Biotech	Phase II/III
AG3340	Agouron/ Hoffman-La Roche	Phase II/III
D2163	Chiroscience/ Bristol Myers-Squibb	Phase I
D1927	Chiroscience/ Bristol Myers-Squibb	Phase I
CGS27023	Novartis	Phase I
BAY12-9566	Bayer	Phase I

that they or their derivatives are also able to block the expression of MMP-2 and MMP-8 in keratinocytes (121), endothelial cells and rheumatoid synovial cells (122), and the expression of MMP-3 in fibroblasts (123). Consequently, the ability of synthetic tetracyclines to inhibit invasion of prostate carcinoma cells has been shown (124). Tetracyclines have also been shown to induce apoptosis of malignant cells, providing further evidence that MMP activity in general may play an important role in the survival of neoplastic cells (124, 125).

Bisphosphonates are a group of pharmacological substances recently identified as MMP inhibitors (126). They were developed as inhibitors of bone resorption and have been used to treat patients with bone metastases (127). Bisphosphonates have also been shown to inhibit secretion of MMP-2 (128) and to prevent the inhibitory effect of TIMP-2 on MMP-2 degradation by plasmin and thereby enhance the inactivation of MMP-2 (129).

Conclusion

In conclusion, there is convincing evidence that MMPs play an important role in the invasive capacity and growth of various malignant tumours and that the expression of certain MMPs can be used to estimate the metastatic capacity and prognosis of malignant tumours. In addition, there is evidence that MMP activity may also play a role in the survival of malignant cells. The ongoing clinical trials with synthetic MMP inhibitors are expected to show whether the concept of MMP inhibition has a place in the therapeutic arsenal aimed at inhibiting growth, invasion and metastasis of malignant tumours.

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MATRIX METALLOPROTEINASES AND THEIR INHIBITORS IN TUMOUR GROWTH AND INVASION

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Matrix Metalloproteinases and Coronary Artery Disease: A Novel Therapeutic Target

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Matrix metalloproteinases (MMP) are a family of enzymes that selectively digest individual components of the extracellular matrix. Their function has been studied in both normal physiologic processes and pathologic states. In the blood vessel, MMPs play an important role in maintaining the vessel's integrity by breaking down extracellular matrix while new matrix is being synthesized. This is necessary to avoid weakening from continuous mechanical stresses. However, in certain environments, these MMPs may contribute to cardiovascular pathologic processes. The purpose of this review is to first discuss the role of MMPs in coronary vascular disease. Evidence suggests that MMPs contribute to the development of de novo atherosclerotic plaques and postangioplasty restenotic plaques by allowing smooth muscle cells to migrate from the vascular media to the intima. Evidence also suggests that MMPs contribute to the rupture of these plaques by degrading the fibrous cap that surrounds them. With this increased molecular information that concerns the pathogenesis of coronary vascular disease, new molecular therapies aimed at altering these processes are being investigated. The rationale, mode of delivery, and prospects for success of these therapies will also be discussed here. J Clin Pharmacol 1997;37:991-1000.

Matrix metalloproteinases (MMPs) are a family of enzymes that are important in the resorption of extracellular matrices in both normal physiologic processes and pathologic states (Table I). Individually, these enzymes digest selective components of the extracellular matrix. Collectively, this family can degrade the entire extracellular matrix.

In the past few years, MMPs were mostly studied in the context of normal physiologic development, postnatal remodeling, and in the pathologic resorption associated with invasive tumors, periodontal disease, and rheumatoid arthritis. More recently, this area of study expanded to include the cardiovascular system. Evidence now suggests that MMPs play an important role in maintaining blood vessel integrity.¹⁻³ To avoid weakening from the normal mechanical stresses of blood pressure, a vessel must continuously remodel its connective tissue. Matrix metalloproteinases contribute to this remodeling by break-

ing down the extracellular matrix while new matrix is synthesized.

Matrix metalloproteinases may also play an important role in pathologic states in the coronary artery system. By breaking down the extracellular matrix, MMPs may allow smooth muscle cells to invade and migrate, which contributes to pathologic processes such as atherosclerosis and postcoronary angioplasty restenosis. In addition, MMPs may degrade the fibrous cap of an atherosclerotic plaque, thereby contributing to coronary plaque rupture. This review discusses the role of MMPs in coronary artery disease and explains possible therapeutic approaches through manipulation of the MMP system.

THE MATRIX METALLOPROTEINASE FAMILY

The MMP family has been subdivided into three groups, based on substrate preference: the collagenases, gelatinases, and stromelysins (Table II). A fourth group made up of integral membrane proteins has recently been elucidated. At present, each MMP can be referred to by either its molecular weight, its order of identification, or its substrate specificity. This review will use substrate specificity.

The MMP family of enzymes are calcium activated.

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TABLE I

Matrix Metalloproteinase Involvement in Tissue Resorption/Degradation	
Normal Process	Pathological Process
Ovulation	Cancer invasion
Endometrial cycling	Tumor metastasis
Blastocyst implantation	Rheumatoid arthritis
Embryogenesis	Osteoarthritic cartilage
Salivary gland morphogenesis	Periodontal disease
Mammary development/ involution	Wound/fracture healing
Cervical dilatation	Fibrotic lung disease
Fetal membrane rupture	Liver cirrhosis
Uterine involution	Corneal ulceration
Bone growth plate	Gastric ulcer
Bone remodeling	Dilated cardiomyopathy
Angiogenesis	Aortic aneurysm
Tooth eruption	Atherosclerosis
Hair follicle cycle	Otosclerosis
Macrophage function	Epidermolysis bullosa
Neutrophil function	

From Woessner JF Jr.: The family of matrix metalloproteinases. *Ann NY Acad Sci* 1994;732:14, with permission.

zinc endopeptidases that share similarity at the amino acid level. These enzymes have several structural features in common, including a pro-peptide domain that contains a cysteine switch⁴, a catalytic zinc binding domain, and a hemopexin-like domain.^{5,6}

Production and Regulation

Matrix metalloproteinases can be secreted from many cells including immune cells, fibroblasts, tumor cells, endothelial cells, smooth muscle cells, and foam cells.⁷⁻⁹ Their synthesis and activity must be closely regulated to prevent any over- or under-production that may lead to excessive tissue destruction or a fibrotic process. To maintain tight control, MMPs are regulated at three main levels: transcription, activation of latent proenzymes, and inhibition of activity by endogenous inhibitors called tissue inhibitors of MMPs.

The regulation of MMP genes in normal tissue is not thoroughly understood at present. Complicating this area of research are differences between *in vitro* and *in vivo* patterns of expression, variation of regulation in different tissues, and evidence of both inducible and constitutive expression of certain MMPs. Individual MMP expression will be discussed below.

All members of the MMP family are produced as

inactive zymogens that must be cleaved to become active.^{10,11} These proteases are thought to be held in the inactive form through coordination of a cysteine in the proregion with the active site zinc ion, thereby blocking access to the active site.¹² Activation of most MMPs involves cleavage of the propeptide which destabilizes this cysteine-zinc interaction. Plasmin and stromelysin are known physiologic activators.^{11,13} However, Progelatinase A lacks the appropriate protein cleavage site required for enzyme activation.¹⁴ Other mechanisms of activation have been described for gelatinase A, including self activation^{11,15} and receptor-mediated activation.^{11,16,17}

Once the zymogen is activated, the major point of control lies with the tissue inhibitors of MMP (TIMP). To date there are three members of the tissue inhibitors family: TIMP-1, TIMP-2, TIMP-3. All three types contain two domains: the N-domain which reacts with the active site of MMPs, and the C-domain which binds to other components of the MMP.¹⁸ The inhibitor TIMP-1 is synthesized by macrophages and most connective tissue cells. It acts against all members of the MMP family and is highly inducible by cytokines and hormones. The inhibitor TIMP-2 acts more specifically on gelatinase A (MMP-2), and its expression usually follows that of gelatinase A. This inhibitor, along with its substrate, is generally found at a constant level in vascular connective tissue and it is not easily induced. The inhibitor TIMP-3 is anchored in the matrix, and has the additional properties of stimulating cell growth, but is not as well understood.¹⁹

A fourth regulatory mechanism is being studied that involves a negative feedback system. Some research has suggested that plasmin can both activate MMPs and also provide a negative feedback for its degradation response.²⁰ At low levels, plasmin induces the synthesis and activation of collagenase and stromelysin from smooth muscle cells.²¹ However, plasmin in high concentrations will induce plasminogen activator inhibitor-1 secretion from smooth muscle cells. This induction may act as a negative feedback by limiting further plasmin generation, and therefore reduce further synthesis and activation of MMPs. Proposed mechanisms for plasminogen activator inhibitor-1 induction include mechanical cytoskeletal changes on the smooth muscle cell from surrounding extracellular matrix breakdown²² or a receptor-mediated mechanism by extra-cellular matrix breakdown products.

THE METALLOPROTEINASES IN CORONARY ARTERY DISEASE

Atherosclerosis

The classic mechanism for the pathogenesis of atherosclerosis, called the "response to injury hypothe-

TABLE II

MMP Subgroups

Subgroup	Name	MMP #	Molecular Wt. (Latent Form) (kD)	Substrate	Regular in Human VSMCs	Native Inhibitor
Collagenases	Interstitial C	1	55	C types III, I, II, VII & X; Gelatin, proteoglycans	IL-1, TNF α , PDGF, phorbol	TIMP-1, -2
	Neutrophil C	8	75	C types I, III, II, proteoglycans		TIMP-1, -2
Gelatinases	Gelatinase A	2	72	C types IV, V, VII & X; gelatin	constitutive	TIMP-2, -1
Stromelysins	Gelatinase B	9	92	C types IV, V, VII & X		TIMP-1, -2
	Stromelysin 1	3	57	C types III, IV, V & IX; laminin, fibronectin, elastin, gelatin, proteoglycans, progelatinase B, procollagenase	IL-1, TNF α	TIMP-1, -2
	Stromelysin 2	10	57	Same as above		TIMP-1, -2
	Stromelysin 3	11	51	Gelatin, fibronectin, proteoglycans		TIMP-1, -2
	PUMP-1	7	28	Gelatin, fibronectin, laminin, C type-IV, procollagenase, proteoglycan core protein		TIMP-1, -2
	Matrilysin	12	57	Elastin		TIMP-1, -2
Membrane-	MT-MMP	-	66	C type IV, gelatin, progelatinase A		TIMP-1, -2

VSMC, vascular smooth muscle cells; C, collagen; IL-1, interleukin-1; TNF α , tumor necrosis factor alpha; TIMP, tissue inhibitor of matrix metalloproteinase.

Bold type denotes a potent interaction between the compounds indicated.
From Ref. 13 with permission.

sis" states that atherosclerosis is initiated as a response to arterial endothelial injury, mechanical or functional, which allows increased permeability to lipids and monocytes and permits platelets to adhere to the endothelium.²³ The monocytes then transform into macrophages in the intima and accumulate lipids to become foam cells. These macrophages and platelets at the surface then release multiple factors, which include interleukin-1, tumor necrosis factor, and platelet-derived growth factor, and may cause migration of medial smooth muscle cells into the intima to proliferate and produce the extracellular matrix of the resulting lesion.

It is proposed that the mechanism that medial smooth muscle cells migrate to the intima is mediated by MMPs degrading the extracellular matrix surrounding them and the basement membrane separating the media from the intima. This would involve the breakdown of fibronectin and collagen type I found in the extracellular matrix, and collagen type

IV, laminin and heparin sulfate proteoglycans, found in the basement membrane. It has been established that the cytokines secreted by the intimal macrophages can stimulate smooth muscle cells *in vitro* to produce MMPs that are capable of degrading these substances.⁹

Human smooth muscle cells in culture respond to interleukin-1 and tumor necrosis factor alpha by increasing the secretion and the activity of stromelysin (MMP-3), interstitial collagenase (MMP-1), and gelatinase-B (MMP-9). Stromelysin, among other things, activates pro-collagenase and progelatinase B. Interstitial collagenase acts by disrupting the native structure of collagen I fibrils found in the media. These degraded fibrils can then be further degraded by either gelatinase A or B (MMP-2 or 9, respectively).

Gelatinase A (MMP-2), TIMP-1, and TIMP-2 also were produced by these smooth muscle cells before and after stimulation, but their concentration was

unaffected by the stimuli. Gelatinase A is the MMP that degrades the collagen found in the basement membranes. *In vitro* studies have shown that smooth muscle cell invasion through basement membrane is dependent on production of gelatinase A and can be inhibited by gelatinase A inhibitors.²⁴ Although gelatinase A did not increase in concentration after stimulation by interleukin-1 or tumor necrosis factor- α , it was found in a lower molecular weight and a more active form after stimulation.⁹ As mentioned previously, there is usually a constitutive secretion of gelatinase A and its activation is different from the other MMPs. Perhaps this MMP does not increase in concentration in response to stimulation, but becomes more active. Regardless, the ratio of MMP activity to tissue inhibitors of MMP increased after interleukin-1 and tumor necrosis factor- α stimulation to the smooth muscle cell, favoring the necessary connective tissue and basement membrane breakdown for smooth muscle cell migration from the media to the intima.

Other *in vivo* evidence has connected MMPs with the atherosclerotic process. A common polymorphism in the promoter region for stromelysin has been associated with a faster progression of coronary atherosclerotic disease in humans.²⁵ Stromelysin acts on many substrates, including proteoglycans, collagen II, IV, and IX, laminin, fibronectin, and gelatin.²⁶⁻²⁹ When 72 patients from the St. Thomas Atherosclerosis Regression Study (STARS) were studied, patients with an altered stromelysin promoter region had a more rapid progression of coronary atherosclerotic disease than those without this altered genotype. It is proposed that MMPs and tissue inhibitors of MMPs are coordinately regulated in response to cytokines, and thus a change in the transcription of the enzyme and not the inhibitor may disrupt the normal balance. Therefore, extracellular matrix degradation would predominate and smooth muscle cells could migrate faster.

This significant difference, however, was only found in comparing those with the least stenosis (<20%) and those with the highest serum low-density lipoprotein cholesterol (plasma LDL >4.2 mmol/L). This finding of significant progression in the least stenosed vessels is consistent with several other studies that showed a more rapid progression in less stenosed vessels.³⁰ It is possible that those patients with more severe stenosis were progressing too slowly to detect a significant difference between the rates. The association between low-density lipoprotein cholesterol and cardiovascular disease has been documented in many studies. Interestingly, patients with the altered genotype who were treated with diet and lipid-lowering agents did not exhibit the same

rapid progression effects. It is proposed that products of the oxidized low-density lipoprotein produced in the intima may alter cells to produce more cytokines, thus stimulating more MMP production and smooth muscle cell migration, which facilitates atherosclerosis. If oxidized low-density lipoprotein induces this reaction in all patients, possibly patients with this altered genotype have an enhanced response, i.e. a genetic predisposition to progress faster.

Angioplasty Restenosis

Percutaneous transluminal coronary angioplasty is a widely used treatment for angina pectoris. It is an attractive procedure because of its limited invasiveness and minimal complication rate compared with surgery. However, its usefulness is limited by a high restenosis rate.

The pathogenesis of human restenotic lesions after percutaneous transluminal coronary angioplasty is not well defined, but there is some consensus regarding the general sequence of events.³¹ Within the first 24 hours, "recoil" can occur secondary to vessel elasticity,^{32,33} with thrombi formation subsequent to exposure of subendothelial structures to blood. Because atherosclerotic lesions vary in composition, their thrombotic potential does as well. Evidence suggests that the highly lipid plaques are the most thrombogenic.³⁴

The involvement of smooth muscle cells in this event is less clear. Evidence suggests medial smooth muscle cells immediately replicate on day 1³⁵, migrate to the intima on day 4, and then may replicate again in the intima and produce an extracellular matrix.³⁶ As discussed in the development of atherosclerosis, MMPs may play a central role in migration of medial smooth muscle cells to the intima. This theory is supported by a rise in gelatinase B synthesis and activity in the vessel 1 day after balloon injury, and its continued presence for 6 days after injury.³⁷

However, smooth muscle cells in an artery post-percutaneous transluminal coronary angioplasty are in a strikingly different environment than they were in the development of the *de novo* atherosclerotic plaque. In injured arteries there are less intact cells and intimal smooth muscle cells are present from the original atherosclerotic plaque; there are different degrees of thrombus depending on the plaque make-up; and different cytokines and chemoattractants. Therefore, smooth muscle cells in this postangioplasty environment probably act differently. Evidence suggests smooth muscle cells in an environment similar to a balloon injured vessel produce less TIMP-1 and 60% more active gelatinase A without any stimulation.²⁴ In addition, these smooth muscle

cells will migrate and invade a basement membrane with platelet-derived growth factor added to the medium, whereas smooth muscle cells in an intact vessel environment will neither migrate nor invade without additional stimulation.

The significance of these smooth muscle cells to platelet-derived growth factor is unclear. Platelet-derived growth factor is a potent mitogen and chemoattractant for smooth muscle cells. It is secreted from platelets, endothelium, smooth muscle cells, and macrophages,³⁸⁻⁴⁰ and it seems to play a role in post-angioplasty restenosis. Evidence has shown that balloon catheter arterial injury in rats induces expression of both platelet-derived growth factor and its receptor in the resulting neointimal lesion⁴¹. In addition, administration of an anti-platelet-derived growth factor antibody before and after balloon artery injury reduces the thickness and cellular content of the neointima lesion by nearly 41%,⁴² while infusion of platelet-derived growth factor for 7 days increases the intimal lesion 15 fold.⁴³

Interestingly, when an MMP inhibitor is added to the injury vessel environment where smooth muscle cells react to platelet-derived growth factor, invasion of the basement membrane is inhibited but migration is not. Therefore, invasion of the basement membrane in the postangioplasty state seems to be MMP-dependent, but the migration of smooth muscle cells is not, even though both smooth muscle cell migration and basement membrane invasion are triggered by platelet-derived growth factor in this environment.

In addition, one has to question if the migration of medial smooth muscle cells is absolutely necessary to form a restenotic plaque because intimal smooth muscle cells are already present from the original plaque. *In vivo* animal studies support this idea by showing that when an MMP inhibitor is given before and after balloon catheter injury for up to 14 days, the number of intimal smooth muscle cells was greatly reduced, but not completely obliterated.⁴⁴ This reduction correlated to an initial reduction in intimal thickening at 7 to 10 days. However, eventually it seems that the small number of smooth muscle cells that did cross the basement membrane were able to increase proliferation to compensate. At day 14, the intimal lesion had the same cell number and size as controls. Therefore, it is possible that the small number of intimal smooth muscle cells present after percutaneous transluminal coronary angioplasty could also compensate by proliferating even if all migration of medial cells is inhibited.

The role of plasminogen in MMP regulation has already been addressed and is now being studied in the context of restenosis. Both *t*PA and *u*PA are

expressed in smooth muscle cells after arterial injury. Expression of *U*-PA in smooth muscle cells is detectable immediately after arterial balloon injury, and *t*PA is detectable at 3 days, around the time of smooth muscle cell migration.⁴⁵ In an attempt to link plasmin with smooth muscle cell migration, rats infused with a compound which inhibits plasmin production showed a significant reduction in the rate of smooth muscle cell migration in ballooned arteries.^{46,47} This migration could be secondary to either direct matrix degradation by plasmin or by activating MMPs.

Another study has shown that treatment with a heparin fraction (low molecular weight and no anticoagulant function) for 14 days after balloon injury in rats decreased *t*PA but not *u*PA, and resulted in a 60% reduction in smooth muscle cell accumulation and intimal thickening.⁴⁸ By inhibiting *t*PA, heparin may be inhibiting the activation of plasminogen that is required for activation of many latent MMPs, although it is not clear why *u*PA would not make up for the lost *t*PA function. Alternatively, heparin inhibits collagenase at the level of transcription.⁴⁹ This evidence supports the idea that plasmin/plasminogen activators are important in restenosis, however, the molecular mechanism for these effects are unknown and may be unrelated to MMPs. More research needs to be performed examining this relationship between *t*PA and intimal smooth muscle cell accumulation.

The role of tissue inhibitors of MMP in atherosclerosis has not been as well studied as MMPs. However, it has been noted that TIMP-1 synthesis is greatly elevated in the neointima 8 weeks after endothelial removal in a rabbit aorta.⁵⁰ This elevation is not well understood. It is probable that tissue inhibitors of MMP have functions other than inhibiting MMPs, and may contribute to the development of atherosclerosis. Hayakawa et al⁵¹ demonstrated evidence that tissue inhibitors of MMP promote fibroblast and endothelial cell proliferation. More research in this area is needed as well.

Rupture of Coronary Atherosclerotic Plaques

Rupture of coronary atheromas accounts for most acute myocardial infarctions. These rupture-prone areas (shoulder and core) generally contain accumulations of activated macrophage foam cells and T lymphocytes with few smooth muscle cells present.⁵²⁻⁵⁴ These areas also contain reduced collagen and glycosaminoglycan concentrations.

It is suggested that MMPs derived from macrophages may digest the extracellular matrix in the fibrous cap, leading to plaque rupture. *In vitro* studies have certainly shown that macrophages are able to

break down fibrous caps and this degradation is correlated to the presence of MMP secretion.⁵⁵ In addition, all three subgroups of the MMP family have been identified intracellularly in macrophages accumulated in rupture-prone areas of human atherosclerotic plaques.⁵⁶ This intracellular location indicates active synthesis because MMPs are not stored.

Other studies have only been able to associate gelatinase B with rupture-prone areas *in vivo*. When athrectomy specimens obtained from patients with unstable angina were compared with specimens from patients with stable angina and normal internal mammary arteries, it was found that gelatinase B was present in 83% of the patients with unstable angina, 25% of the patients with stable angina, and 0% in the normal internal mammary arteries.⁵⁷ Again, gelatinase B enzyme was found intracellularly in the macrophages. This study shows a strong association between the presence of gelatinase B in human coronary atherosclerosis with the clinical syndrome of unstable angina. It is possible that only 83% of the unstable angina patients were positive because of sampling error inherent in athrectomy procedures and 25% of the stable angina patients were positive because they were going to progress to unstable angina.

It is unclear at present which MMPs are involved in coronary plaque rupture and what activates their production. However, because activated macrophage foam cells and T lymphocytes are the dominant cells in vulnerable regions, the interaction between these two cells may be critical to the process of rupture. One study has demonstrated that T lymphocytes that express high levels of CD40 ligand gp39 can stimulate human monocytes to produce gelatinase B.²¹ Although macrophages and T lymphocytes are present all over plaques, their accumulation in vulnerable areas and the presence of few smooth muscle cells make these areas most prone to degradation.

Other mechanisms of stimulating MMP production in these areas have been proposed as well. It seems that macrophage foam cells from atherosclerotic lesions secrete stromelysin and collagenase without any stimulation, whereas nonlipid laden macrophages do not.⁵⁸ It is possible that the reactive oxygen radicals and oxidized low-density lipoprotein generated from these foam cells stimulate macrophage secretion of MMPs. Therefore, multiple mechanisms of how MMPs could produce rupture in prone areas of atherosclerotic plaques exist. It is probable that different combinations of these mechanisms contribute to each rupture event.

POTENTIAL THERAPEUTIC INTERVENTIONS

The potential for drug therapy to intervene in the actions of MMPs to treat cardiovascular disease is

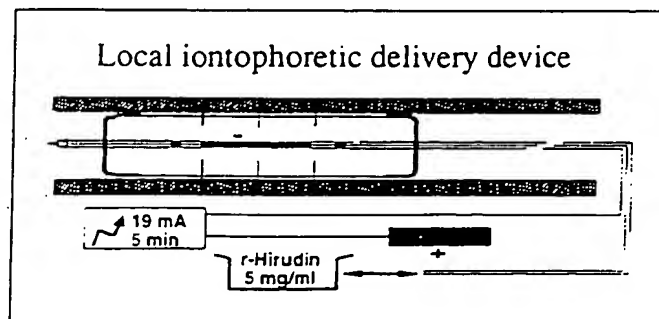


Figure 1: Schematic representation of the iontophoretic approach to local drug delivery.

real. However, target specificity and selectivity will be of primary importance in developing new treatments in this area. As seen in Table I, MMPs are involved in tissue resorption and degradation all over the body. Thus, new drugs must act in specified local areas and avoid systemic effects.

Novel ideas for the delivery of these potential treatments are being examined. Promising clinical applications include temporary or permanent local delivery devices within the vasculature to enhance bioavailability and target specificity.³¹ Some experiments have shown encouraging results with local drug delivery catheters that use an iontophoretic mechanism to facilitate delivery. This device uses electrical currents to enhance the movement of charged molecules through tissue, and results in an even distribution of the drug without significant endothelial damage.⁵⁹ To avoid distal ischemia, a reperfusion lumen catheter is also used (Figure 1).

Another mechanism for achieving high local concentrations in a vessel uses a barophoretic approach.¹² This device uses a double-balloon catheter creating a minienvironment isolated from blood between the balloons where therapeutic agents can be delivered (Figure 2). To avoid distal ischemia, this device also has an internal conduit for the continued

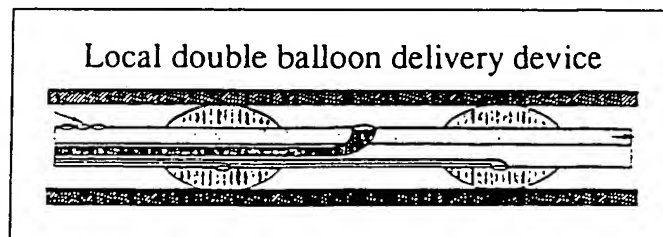


Figure 2: Schematic representation of the barophoretic approach to local drug delivery.

flow of blood. Both of these systems offer a significant increase in specificity and transfer efficiency over systemic delivery or other local devices under investigation.³¹

In addition to method of delivery, the other biggest obstacle is the therapeutic agent to be used. A number of agents have been created to inhibit the synthesis, activation, and active sites of MMPs.⁶⁰⁻⁶² For instance, N-aryl-pyrido-fused isothiazolones are non-peptidic small molecules that seem to inhibit activation of pro-MMPs by binding to Cys75 in the pro-region of the MMP zymogen. In turn, this binding interferes with the normal activation of these proteases (Figure 3). This inhibition has been documented for MMP-3 and is assumed to inhibit the entire MMP family because they share a common activation mechanism.

In considering the development of *de novo* atherosclerotic lesions and their progression, MMPs seem to be most involved in this process by allowing the smooth muscle cells to migrate to the intima. Because this is a slowly developing process that can continue over the course of a lifetime, an intravascular device seems impractical in this situation. For the same reason, a generalized MMP inhibitor given over a systemically long term would probably have far too many toxic side effects to make it useful. In addition, it may prevent plaque reorganization and collateral vessels from forming because evidence

would strongly suggest that MMPs are necessary in neoangiogenesis and plaque reorganization.^{56,63}

A better approach may be one that would block the specific stimulators in vessels that induce MMP transcription or activation during times of atherosclerosis formation. As discussed in the STARS study, the association of an altered MMP gene with progression of cardiovascular disease only became significant when combined with high levels of serum low-density lipoprotein. Therefore, one potential way of preventing MMP induction in vessels is already being used in clinical practice by lowering low-density lipoprotein cholesterol with diet and/or drug therapy.

The problem of restenosis would theoretically be easier to treat due to the acuteness of vessel injury and the isolated location to direct treatment. It is in this situation that an intravascular device may be most beneficial. However, the development of efficacious agents to be used in these devices has failed, mostly due to the paucity of precise information concerning the mechanism of human restenosis. It is probable that development of restenosis utilizes several mechanisms, thus merely blocking one pathway with a drug may initiate compensation by a different pathway.

Experiments discussed showed that inhibition of MMPs did not reduce long-term neointimal lesions.⁴⁴ Perhaps medial smooth muscle cell migration is not the correct target for therapeutics because human

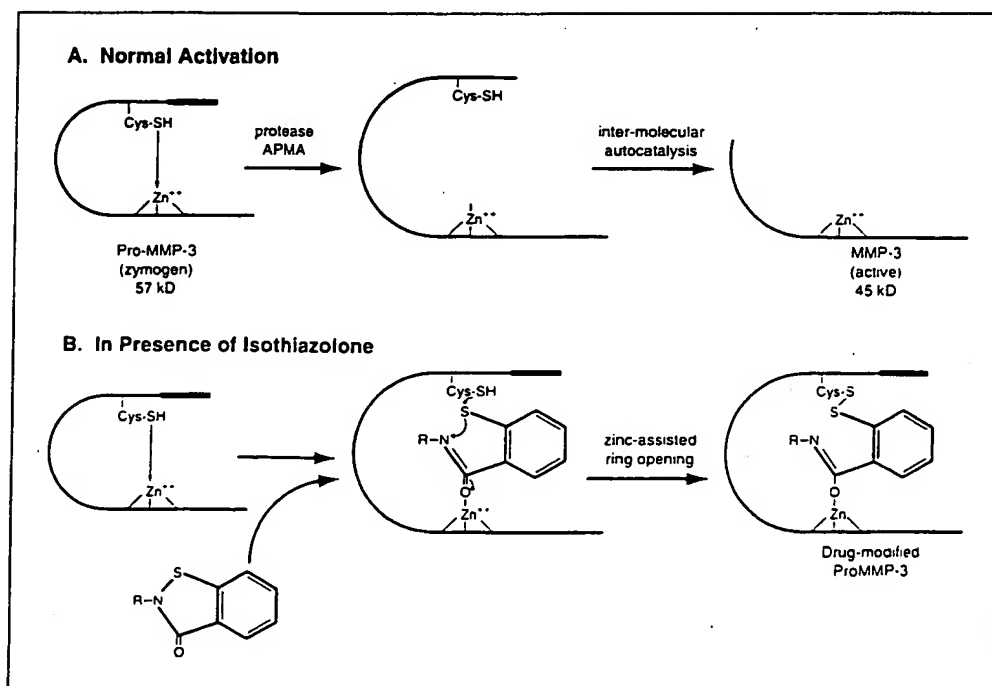


Figure 3: (A) absence of inhibitor, activation of the propeptide involves the dissociation of the cysteine thiol from the zinc ion, which exposes the active site, allowing bimolecular autolysis to occur. (B) isothiazolones react with the cysteine thiol residue, forming a disulfide bond. This adduct can then act as a ligand for zinc, replacing the cysteine thiol and in turn interfering with the normal activation of proMMP-3. Abbrev: APMA = 4-aminophenylmercuric acid. From Ref. 60, with permission.

atherosclerotic arteries undergoing percutaneous transluminal coronary angioplasty already possess intimal smooth muscle cells. It is possible that even with complete suppression of medial smooth muscle cell migration, intimal smooth muscle cells can compensate by replicating and producing the extracellular matrix of a new lesion anyway. A better target may be the prevention of intimal smooth muscle cell proliferation and production of extracellular matrix. Potential investigations in the future may involve temporarily implanting a double-balloon baro-photic catheter with a drug to be delivered to the site of injury that would act on these intimal smooth muscle cells directly after percutaneous transluminal coronary angioplasty.

Therapeutic intervention regarding plaque rupture is complicated by the sudden onset of these events with little warning. Once the plaque has ruptured, intervening with MMPs probably has no effect. Therefore, therapeutic intervention should be aimed at preventing what stimulates macrophage foam cells from synthesizing MMPs that may initiate rupture. Two areas could address this problem, and prevention of plaque rupture may need to utilize both of them. One would involve the prevention of T lymphocytes from activating macrophages to secrete MMPs. This has been done *in vivo* by giving an anti-gp39 antibody to prevent arthritis in mice. However, its long-term effects in humans are unknown. The second proposal would involve the other proposed trigger of MMP secretion by macrophages, oxidized low-density lipoprotein. Again, we are already routinely lowering low-density lipoprotein in patients.

These are only a few ideas for developing treatments to intervene in the role MMPs play in cardiovascular disease. Further research will surely make this an important and useful target for pharmacotherapy.

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ERRATUM

In the article "Dependency of Cortisol Suppression on the Administration Time of Inhaled Corticosteroids," by Bernd Meibohm et al (*J Clin Pharmacol* 1997;37:704-710), equation (6), which appeared on page 706, was printed incorrectly. The correct equation appears as follows:

$$CCS = \frac{V_d^{Cort} \cdot \int_{t_D}^{t_D+24h} R_c \cdot \frac{E_{max} \cdot C_f}{E_{50} + C_f} dt}{12h \cdot R_{max}} \quad (6)$$

Metalloproteinase Inhibition Reduces Thrombolytic (Tissue Plasminogen Activator)-Induced Hemorrhage After Thromboembolic Stroke

Paul A. Lapchak, PhD; Deborah F. Chapman, MSc; Justin A. Zivin, MD, PhD

Background and Purpose—A potentially dangerous side effect associated with tissue plasminogen activator (tPA) use is cerebral hemorrhage. We have focused on developing drugs that could be administered with tPA to reduce the rate of hemorrhage. Since recent studies suggest that various matrix metalloproteinases (MMPs) are important in tumor necrosis factor- α production and membrane and vessel remodeling after ischemia, we investigated whether MMP inhibition affected the rate of hemorrhage and infarct production in the absence or presence of tPA treatment.

Methods—We occluded the middle cerebral artery of New Zealand White rabbits with radiolabeled blood clots. Five minutes after embolization, we administered either the MMP inhibitor BB-94 (30 mg/kg SC) or its vehicle. Additional groups received BB-94 or vehicle in combination with tPA, administered 60 minutes after embolization (3.3 mg/kg tPA). After 48 hours, the rabbits were killed and brains were removed, immersion fixed for 1 week in 4% paraformaldehyde, and then cut into 5-mm coronal sections that were examined for the presence of hemorrhage, infarcts, and recanalization.

Results—Hemorrhage after embolic stroke was detected in 24% of the control group. tPA induced macroscopically visible hemorrhage in 77% of the tPA-treated group. The rabbits treated with BB-94 had an 18% incidence of hemorrhage ($P>0.05$ compared with control). However, when the combination of BB-94 and tPA was administered to rabbits, there was only a 41% incidence of hemorrhage (compared with 77% in the tPA group; $P<0.05$). Both tPA and BB-94/tPA were similarly effective at lysing clots, at 49% and 35% ($P<0.05$), respectively, compared with the 5% rate of lysis in the control group. There was a trend for a reduction in the number of infarcts, but it did not reach statistical significance.

Conclusions—Our data suggest that MMP inhibition attenuates mechanisms involved in tPA-induced hemorrhage. This novel form of combination therapy may show promise as a treatment strategy for acute stroke. (*Stroke*. 2000;31:3034-3040.)

Key Words: cytokines ■ intracerebral hemorrhage ■ ischemia ■ matrix metalloproteinases ■ membranes ■ neuroprotection ■ tumor necrosis factor

Thrombolysis is now gaining increasing acceptance for acute stroke management; however, only a small subset of potentially eligible patients are being treated with tissue plasminogen activator (tPA).¹⁻³ Overall, tPA is quite beneficial, even though there is a small window of opportunity for treatment and a potentially dangerous side effect of hemorrhages.^{2,4-6} A series of trials have shown that thrombolytics alone have limited efficacy,⁷ suggesting that additional treatment strategies are needed. Nevertheless, the finding that at least one acute therapy is effective in reducing neurological damage was an important proof of concept. Even though tPA is efficacious, there is one major shortcoming to the drug. tPA significantly increases the intracerebral hemorrhage (ICH) rate in patients approximately 10 times greater than that observed in placebo-treated controls, and half of the patients who develop symptomatic tPA-related ICH die.¹ Because of

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this serious side effect, emphasis should be placed on continuing to develop new pharmaceuticals that can be used in combination with tPA^{2,8} to make tPA a safer stroke therapy.

Recently, a few research groups have focused on the role of matrix metalloproteinases (MMPs) and non-MMPs in the processing of tumor necrosis factor- α (TNF- α)⁹⁻¹¹ and in cerebral ischemia, edema, aneurysms, and hemorrhage.^{12,13} When the multiple roles of MMPs in the central nervous system (CNS) are considered, it is apparent that they may be involved in both membrane remodeling and the production of cytokines that may be deleterious to neuronal function and vasculature after a stroke. Pharmacological intervention at the level of MMPs may minimize stroke-induced tissue damage and reduce hemorrhage. Thus, we studied whether pharma-

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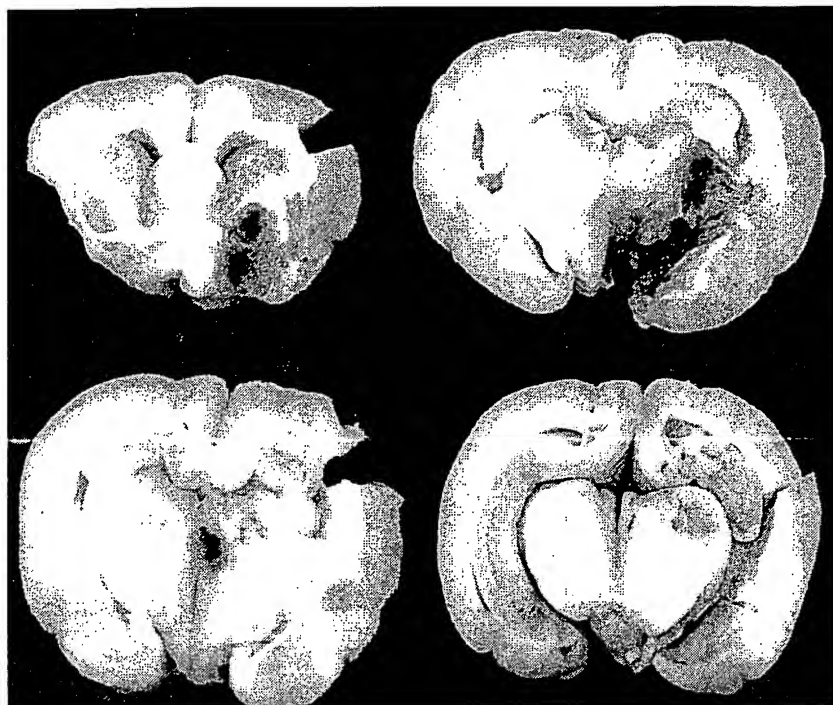


Figure 1. Representative brain sections containing the various types of hemorrhage observed in brain after thromboembolic strokes and thrombolytic treatment. Top left, HI at the level of the septum and caudate putamen. Top right, ICH and HI in the putamen, globus pallidus, and thalamus. Bottom left, ICH in the thalamus. Bottom right, PH in the hippocampus.

cological inhibition of MMPs, with the use of a relatively nonspecific inhibitor, altered hemorrhage rate or conferred neuroprotection in embolized rabbits in the presence or absence of tPA administration.

Materials and Methods

The method we used has been described in detail.¹⁴ Male New Zealand White rabbits weighing 2 to 3 kg were anesthetized with halothane (5% in 3 L/min at induction, 3% in 3 L/min as a maintenance dose). The right internal carotid artery was exposed, and the external carotid artery and the common carotid artery were ligated. If any other branches were seen originating from the internal carotid artery, these were also ligated. A plastic catheter oriented toward the brain was inserted into the common carotid and secured with ligatures. The incision was closed around the catheter so that the distal end was accessible outside. The catheter was filled with 2 mL of heparinized saline (33 U/mL) and plugged with injection caps. The animals were allowed to recover from anesthesia for at least 2 hours before embolization.

Emboli were prepared by withdrawing 1 to 2 mL of arterial blood from a donor rabbit. The blood was mixed with a trace quantity of ⁵⁷Co-labeled plastic microspheres (25 μ m in diameter) and allowed to clot for 3 hours at room temperature. The clot was sliced with a razor blade into small cubes weighing approximately 3 to 4 mg. The cubes were suspended in phosphate-buffered saline containing 0.1% bovine serum albumin. The amount of radiolabel present in each cube was measured with a gamma counter. Just before the embolization, each animal was restrained, and the injection cap of the catheter was removed to allow the rabbit's blood to fill the catheter and wash out the heparinized saline. The line was then filled with heparin-free normal saline. One of the clot cubes was placed inside the injection port of the catheter and rapidly injected with 3 mL of saline flush, followed immediately by a second 3-mL flush. Care was taken during both flushes to ensure that no air bubbles were present in the catheter or syringe. If the animal did not react behaviorally (nystagmus, hemiparesis, seizure) to the embolization, another blood clot was injected in the same way 3 minutes after the first embolization. If there was no behavioral reaction to either embolization, no further blood clots were administered. After the embolization pro-

cess was completed, the catheter was ligated close to the neck, and the rest of the catheter and injection port were cut off.

Animals that died before they were killed were included in this study; the brains were fixed and sectioned as below. The surviving animals were killed 48 hours after embolization. The brains were removed and immersion fixed in 4% paraformaldehyde for at least 1 week and then examined by a blinded observer. The right middle cerebral artery of each brain was examined for the presence of emboli. The surface blood vessels were then stripped from the right hemisphere of each brain and reserved. The cerebellum was also removed from the brain and reserved. The remainder of the brain was cut into six 5-mm-thick coronal slices, each having 2 faces. We noted the presence, location, size, and type of each hemorrhage and infarct. We recorded the size of hemorrhage as the number of section faces showing hemorrhage.^{15,16} Infarction was grossly visible as pale, softer tissue surrounded by pink, normal brain tissue on the brain sections. Three major types of hemorrhage were identified according to the grading system we used in previous experiments. Hemorrhagic infarction (HI) was grossly visible as red speckling of an area, usually surrounded by soft infarcted tissue. Punctate hemorrhage (PH) was characterized by isolated small red marks within the tissue that did not extend through the tissue as a blood vessel would. Parenchymatous ICH was characterized by a large homogeneous mass of blood within the tissue. Examples of each type of hemorrhage are represented in Figure 1. After evaluation for hemorrhage and infarcts, the total radioactivity in the brains was measured by placing the slices into a gamma counter. The surface vessels from the right hemisphere were placed in a separate container. The cerebellum and each hemisphere were then counted in separate tubes. The amount of radiolabel present in the brain (including the right hemisphere vessels) was compared with that contained in the labeled blood clot at embolization. If <10% of the counts were found in the brain and vessels, it was assumed that the labeled blood clot had not reached the brain.^{16,17} The data from these animals were excluded from further analysis. Thrombolysis was defined in 2 ways, by recovery of radioactive label and visual inspection. Any brains containing <20% of the total recovered radioactivity in the surface vessels of the right hemisphere were said to have undergone thrombolysis of the embolus. Then, postmortem, we recorded whether a clot was visible in the middle cerebral artery. This

observation correlated with the recovery of radioactivity in our prior study.^{14,18-20}

Drug Administration

We randomly allocated animals to 4 different treatment groups before the embolization procedure. Sample size was based on power analysis, with $\alpha=0.05$ and $\beta=0.90$, a coefficient of variation of 15%, and a difference between means of 20%. It was determined that a sample size of 12 to 14 animals per group was required. However, our previous experience with this stroke model indicates that we actually need an average of 20 animals, including premature losses caused by various preparation difficulties or deaths after embolization before treatment can be fully administered. The treatments were as follows: tPA (n=60), BB-94 plus tPA (n=26), vehicle (n=28), and BB-94 (n=17). In 2 groups of rabbits, BB-94 or its vehicle was administered subcutaneously 5 minutes after embolization. A fine suspension of BB-94 was freshly prepared in the following vehicle: 0.9% normal saline containing 0.1% PF68 and 0.5% carboxymethylcellulose. BB-94 was administered at a dose of 30 mg/kg on the basis of the recommendation of Dr Helen Mills of British Biotech (Oxford, UK). The recommendation was based on the pharmacokinetic profile of BB-94 after peripheral injection. In the remaining 2 groups of rabbits, we then administered tPA or vehicle 1 hour after embolization. The tPA regimen used in this study was as follows: 3.3 mg/kg tPA, 20% as a bolus injection given over 1 minute, followed by the remainder infused over 30 minutes.^{16,21} Genentech, Inc (South San Francisco, Calif) supplied tPA and its vehicle. tPA was supplied as a lyophilized cake in 50-mg configurations, containing 50 mg tPA (29 million IU), 1.7 mg L-arginine, 0.5 g phosphoric acid, and <4 mg polysorbate 80. The tPA was reconstituted with sterile water for injection, at a concentration of 1 mg/mL. We analyzed the data with the χ^2 test corrected for multiple comparisons, using the Bonferroni technique and ANOVA when relevant.

Results

Stroke Success Rate

Of 131 embolized rabbits included in the study, we found that 84 rabbits (64%) had >10% recovered radioactivity in the brain postmortem. The behavioral manifestations of embolization included nystagmus, pupillary dilation, hemiparesis, or brief, uncoordinated jerking movements. There was a positive correlation between the appearance of abnormal behaviors after embolization, the presence of ^{57}Co in brain resulting from the administered clot, and tissue damage. Even though we monitored the behavioral reaction to the embolus, the strict exclusion criteria were based on the presence of label in brain. The remaining 36% of the rabbits had $\leq 10\%$ of the label present in the brain postmortem, indicating that the injected blood clot did not reach the brain. The breakdown of the 47 rabbits excluded from the study is as follows: vehicle (n=7 of 28; 25%), tPA (n=25 of 60; 41%), BB-94 (n=6 of 17; 35%), and tPA/BB-94 (n=9 of 26; 35%). The rabbits that did not reach the criteria were excluded from the study, and

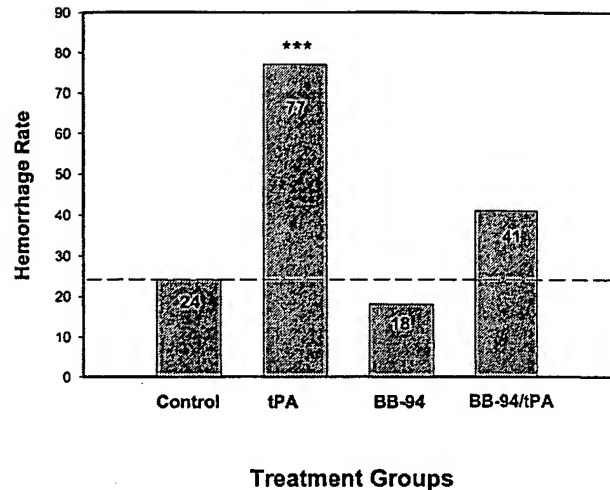


Figure 2. Effect of tPA and BB-94 on hemorrhage rate in embolized rabbits. Hemorrhage rate (percentage) was quantified by counting the number of macroscopically visible hemorrhages in coronal brain sections. tPA administration significantly increased hemorrhage rate compared with either control or BB-94-treated rabbits ($P<0.001$). In rabbits treated with the combination of BB-94 and tPA, there was a significantly lower rate of hemorrhage ($P<0.05$). Results in the BB-94 group were not significantly different from those in the control group ($P>0.05$).

the data were not used in the final analysis. This success rate corresponds well with other studies involving this model.^{17,18}

Types of Hemorrhage After Thromboembolic Stroke

Figure 1 shows 4 coronal brain sections from rabbits after thromboembolic strokes. The top left panel shows an HI in the section at the level of the septum and caudate putamen. The top right panel shows an ICH and an HI in the putamen, globus pallidus, and thalamus. The bottom left panel shows an ICH in the thalamus, and the bottom right panel shows a PH in the hippocampus.

Hemorrhage Rate

Figure 2 shows the hemorrhage rate for the 4 groups of rabbits included in this study. The percentages of rabbits with brain hemorrhages in the 4 groups were as follows: 24% in the tPA/vehicle-treated group (n=21), 77% in the tPA-treated group (n=35), 18% in the BB-94-treated rabbits (n=11), and 41% in those treated with the combination of BB-94 and tPA (n=17). Overall, there was a statistically significant difference in hemorrhage rates (Table). tPA caused significantly more hemorrhages than in the tPA/vehicle control group ($P<0.01$). There was also a significant difference in hemor-

Effect of Pharmacological Treatments on Hemorrhage Types After Thromboembolic Stroke

Treatment Group	Rabbits With No Hemorrhage	Type of Hemorrhage			Total Rabbits With Hemorrhage	n	% Rabbits With Hemorrhage
		PH	HI	ICH			
Control	16	2	3	0	5	21	24
Control+tPA	8	4	23	3	27	35	77
BB-94	9	0	2	0	2	11	18
BB-94+tPA	10	2	6	0	7	17	41

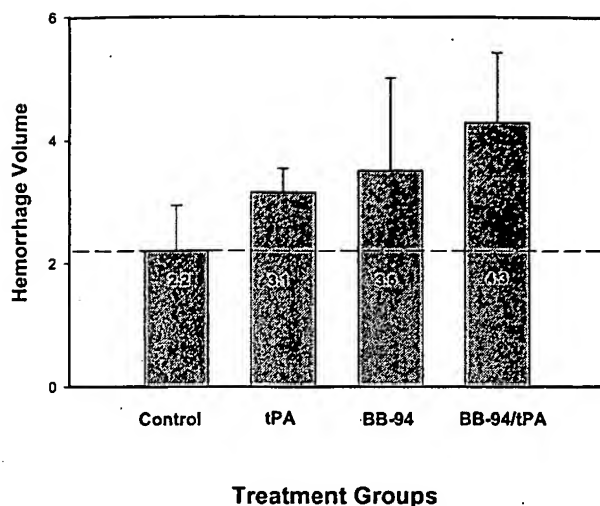


Figure 3. Effect of tPA and BB-94 on hemorrhage volume in embolized rabbits. Hemorrhage volume was quantified by counting the number of faces that show macroscopically visible hemorrhages in coronal brain sections. There were no significant differences among the 4 groups ($P>0.05$).

rhage rate between the BB-94/tPA and tPA groups ($P<0.05$). The drug combination significantly attenuated the rate of hemorrhage production. The hemorrhage rate after a single bolus dose of BB-94 was also statistically different from that of the tPA-treated group ($P<0.05$).

Hemorrhage Volume

The number of faces showing hemorrhage, a qualitative measure of hemorrhage volume, is illustrated in Figure 3. Because 5 brain slices were cut for each rabbit, the maximum number of faces observed was 10. There were no statistically significant differences among the 4 treatment groups. Of the tPA-treated rabbits, there were 3.1 ± 0.4 and 4.3 ± 1.1 faces per hemorrhage for the tPA-treated group and BB-94/tPA-treated groups, respectively. In the tPA-control group, there was an average of 2.2 ± 0.7 faces involved in each hemorrhage, whereas in the BB-94-treated group, there was an average of 3.5 ± 1.5 faces per hemorrhage. By ANOVA, there was no statistical difference between the hemorrhage sizes in any of the treatment or control groups ($P>0.05$). A more detailed analysis of hemorrhage volume using more accurate quantitative methods is required.

Types of Hemorrhage

The Table shows the types of hemorrhage present in each of the experimental groups. Most of the hemorrhages seen were HI, but ICH and PH were also present in each of the groups. Some of the animals had >1 type of hemorrhage present in the brain. For quantitative purposes, we treated each individual hemorrhage observed as a separate entity. Hemorrhages occurred throughout the brain and included the following structures: caudate putamen; thalamus; hippocampus; frontal, parietal, and occipital cortex; hypothalamus; suprachiasmatic area; cerebellum; pons; and midbrain. There were no apparent differences among the groups in the distribution of types or

locations of hemorrhages. In the tPA-treated group, BB-94 decreased the number of HI, PH, and ICH.

Thrombolysis Rate

The main purpose of this series of experiments was to determine the efficiency or efficacy of tPA when a second pharmacological agent was administered. The results are shown in Figure 4. We estimated thrombolytic efficacy by calculating the percentage of animals in each treatment group that had $<20\%$ of the total recovered radiolabel in the surface vessels of the right hemisphere of the brain at postmortem.¹⁸ Thrombolysis was found in 49% of the tPA-treated rabbits (Figure 4), 5% of the tPA/control-treated rabbits, and 35% of the combination drug-treated rabbits. There was no measurable thrombolysis in the BB-94-treated group. There was no significant difference in thrombolysis rate between the tPA and BB-94/tPA groups. However, there were significant differences when comparisons were made between either the tPA or BB-94/tPA groups and the tPA-vehicle control group. There were also significant differences when comparisons were made between either the tPA or BB-94/tPA groups and the BB-94 control group.

Infarct Rate and Volume

In a subset of 2 of the experimental groups used in this study (tPA and BB-94/tPA groups), we determined whether MMP inhibition affected infarct rate and volume (the number of brain slice faces with infarcts) observed in brain after a stroke. In the tPA group, infarcts were found in 94% of treated rabbits (15/16). In the BB-94/tPA group, 65% of the rabbits (11/17) had infarcts. Although there was a trend for BB-94-induced attenuation of infarct rate, the values were not significantly different ($P>0.05$). In the tPA-treated group

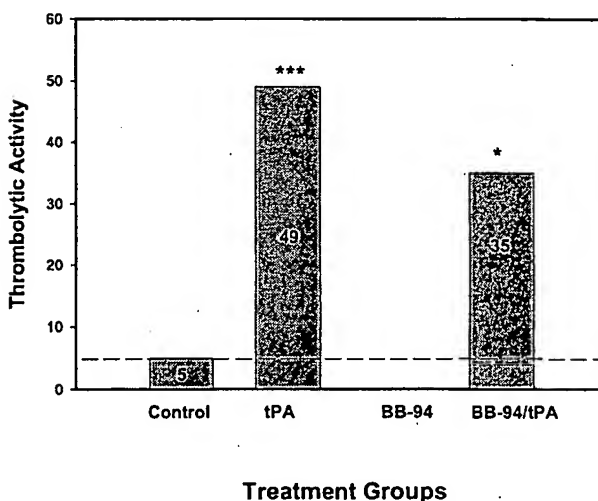


Figure 4. Effect of tPA and BB-94 on thrombolytic activity, shown as percent lysis in embolized rabbits. We estimated thrombolytic activity by calculating the percentage of rabbits in each treatment group that had $<20\%$ of the total recovered radiolabel in the surface vessels of the right hemisphere. There was no significant difference in thrombolysis rate between the tPA and BB-94/tPA groups. Both groups were significantly different from control and from the BB-94-treated group. (** $P<0.001$, * $P<0.05$ compared with control).

there was an average of 3.2 ± 0.6 faces involved in each infarct, whereas in the BB-94/tPA group there was an average of 5.1 ± 0.8 faces per infarct. There was no statistical difference between infarct volumes measured in the 2 groups ($P=0.073$). A more detailed quantitative assessment of infarct volumes would conclusively determine whether BB-94 affects infarct volumes.

Discussion

In the present study we found that the MMP inhibitor BB-94 effectively attenuated the rate of tPA-induced hemorrhage. However, BB-94 did not significantly alter the hemorrhage rate in the absence of tPA administration. In our model the most common type of hemorrhage observed is HI, the remainder being ICH and PH. HI is the most predominant type of hemorrhage in stroke patients, with primary ICH (8% to 15%) constituting most of the remainder.²²⁻²⁴ Thus, the thromboembolic model allows for representation of the types of hemorrhage observed in humans.

The observation that BB-94 inhibited the tPA-induced hemorrhage rate, but not the hemorrhage rate observed in controls, may be due to the low incidence of hemorrhage after a thromboembolic stroke in the absence of tPA. In only approximately 25% of the embolized rabbits do we observe hemorrhage. Thus, a treatment group in the range of 15 to 20 rabbits is too small to confidently conclude that BB-94 alone affected hemorrhage rate. However, since BB-94 reduced tPA-induced hemorrhage, our results suggest that BB-94 effectively inhibits CNS MMP activity after subcutaneous injection. However, BB-94 did not appear to affect hemorrhage volumes measured by the qualitative slice method described in this study. It is possible that a more accurate assessment of hemorrhage volumes could be determined by quantitative methods. Because the thrombolysis rate was not significantly different between the tPA-treated and BB-94/tPA-treated groups, it appears that the reduction of hemorrhage rate was not associated with inhibition of tPA activity in vivo. Our results showing that BB-94 reduced the hemorrhage rate are consistent with previous studies which suggested that MMPs may be important in blood-brain barrier and vasculature function and extracellular matrix remodeling after a stroke.^{25,26} For example, Romanic et al²⁷ used a permanent middle cerebral artery occlusion model in the rat to show that MMP-2 and MMP-9 were increased in neutrophils, endothelial cells, and macrophages soon after stroke. They also showed that systemic administration of neutralizing antibodies to MMP-9 appeared to reduce brain injury after middle cerebral artery occlusion, suggesting that this MMP-9 is involved in neuronal damage after a stroke.²⁷ The observation that MMP-9 is increased in endothelial cells suggests that MMP-9 may be involved in vasculature remodeling and weakening. Investigation of the gelatinases MMP-2 and MMP-9 in a nonhuman primate middle cerebral artery occlusion/reperfusion model showed that MMP-2 was significantly increased soon after stroke, whereas MMP-9 was only increased in subjects with hemorrhagic transformation.²⁸ Bruno et al²⁹ found a correlation between MMP-1 and MMP-2 and matrix remodeling. Overall, the studies suggest that at least 2 MMPs may be directly involved in the

progression of stroke and hemorrhage, specifically MMP-2 and MMP-9. Pharmacological inhibition of MMPs with a nonspecific inhibitor has also previously been shown to reduce edema in a rat collagenase model.³⁰

Regarding infarct rate and volume, we observed that BB-94 administration before tPA produced a trend for a reduction in infarct rates, which suggests that MMPs may also be involved in the ischemic response after embolization. This is in agreement with the findings of Romanic et al.²⁷ However, although the rate of infarcts was slightly reduced, that is, there were fewer sites where infarcts were observed, there was a trend for larger areas of ischemic damage in the presence of BB-94 compared with tPA treatment. The reasons for this apparently contradictory finding require additional study and a more detailed quantitative assessment of infarct volumes and studies aimed at understanding the exact roles of MMPs in tissue damage after stroke.

Additionally, MMPs have been shown to be involved in the production of cytokines in the CNS. Gearing et al⁹ first demonstrated that the mature TNF- α precursor protein could be cleaved to biologically active TNF- α by several MMP enzymes, including the collagenase MMP-1, gelatinases MMP-2 and MMP-9, and stromelysins MMP-3 and MMP-7.^{31,32} MMP-2 and MMP-9 have previously been shown to be active in the processing of pro-TNF- α to TNF- α .^{9-11,33} The authors also showed that specific MMP inhibitors such as BB-2284 could block the production of biologically active TNF- α .⁹ In addition to TNF- α being produced via an MMP, TNF- α can also induce MMPs (ie, MMP-9) in the CNS.³⁴⁻³⁶ This perpetuates the production of MMPs, enzymes that may be deleterious to CNS vessels and membranes. Our findings with the nonspecific MMP inhibitor BB-94, which inhibits MMP-9, suggest that TNF- α production may mediate certain aspects of damage after thromboembolic stroke. Additional studies with more specific MMP inhibitors are required to delineate the role of various MMPs in stroke and in tPA-induced cerebral hemorrhage.

Overall, our study is the first to show that effective combination drug treatments can be developed as novel treatments for stroke. In the present study preadministration of the MMP inhibitor BB-94 significantly reduced the tPA-induced hemorrhage rate and attenuated the brain infarct number. Thus, in effect, the administration of BB-94 improved the safety of tPA by reducing a side effect of tPA.

Acknowledgments

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Editorial Comment

Tissue plasminogen activator (tPA) is the only therapeutic agent approved by FDA for treating ischemic stroke. In the NINDS study, tPA treatment group had a 10-fold increase in symptomatic intracerebral hemorrhage.¹ The increased hemorrhage rate has substantially curtailed the application of tPA in treating patients with acute ischemic stroke. tPA has to be given in strict adherence to the treatment protocol based on the NINDS study to achieve the desirable benefit against the hemorrhagic risk. It has been estimated as few as 2% of all patients with ischemic stroke have received tPA in this country. Any measure that may reduce the incidence of intracerebral hemorrhage associated with tPA may broaden

its clinical use. In the preceding article by Lapchak and associates, a matrix metalloproteinase (MMP) inhibitor, BB-94, was found to be effective in reducing the hemorrhage rate in a thromboembolic stroke model in rabbits. MMPs, having been shown to cause the disintegration of vasculature,² are expressed after cerebral ischemia.³ Thus, inhibition of MMPs is an attractive therapeutic strategy to prevent intracerebral hemorrhage after tPA therapy. Results presented by Lapchak et al are encouraging and suggest that therapeutic attempts to reduce hemorrhage rate after tPA treatment should be further advanced in preclinical and then clinical studies. Results from the present study also raised a number of questions that

deserve further investigation. The first is the failure of BB-94 to reduce the hemorrhage volumes. Because the method used by the group was a rather crude one, quantitative assessment of hemorrhage volumes are needed in future studies. Since BB-94 did not appear to reduce infarct volumes, the reduced hemorrhage rate could not be attributed to its potential neuroprotective effects. Factors other than MMPs, however, may also contribute to tPA-induced intracerebral hemorrhage. One plausible mechanism is reperfusion injury of the ischemic vascular bed. Free radical spin-trapping has been shown to reduce the risk of intracerebral hemorrhage in rat models of stroke.⁴ Together, these findings raise the hope that therapeutic measures that prevent structural disintegration and/or reperfusion injury of the ischemic vascular bed may be developed to broaden the clinical use of tPA in ischemic stroke.

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Matrix Metalloproteinases Collagenase-2, Macrophage Elastase, Collagenase-3, and Membrane Type 1-Matrix Metalloproteinase Impair Clotting by Degradation of Fibrinogen and Factor XII*

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The effects of plasma proteins on controlling the activity of matrix metalloproteinases (MMPs, matrixins) have been the focus of numerous studies, although only a few have examined the influence of matrixins on plasma proteins. Recently, it has been shown that MMPs may play a role in the degradation of fibrin. We have now investigated the role of collagenase-2 (MMP-8), macrophage elastase (MMP-12), collagenase-3 (MMP-13), and membrane type 1-matrix metalloproteinase (MT1-MMP, MMP-14) in the degradation of fibrinogen and Factor XII of the plasma clotting system. Our data demonstrate that the catalytic domains of MMP-8, MMP-12, MMP-13, and MMP-14 can proteolytically process fibrinogen and, with the exception of MMP-8, also inactivate Factor XII (Hageman factor). We have identified the amino termini of the major protein fragments. Cleavage of fibrinogen occurred in all chains and resulted in significantly impaired clotting. Moreover, rapid proteolytic inactivation of Factor XII (Hageman factor) by MMP-12, MMP-13, and MMP-14 was noted. These results support the hypothesis of an impaired thrombolytic potential of MMP-degraded Factor XII *in vivo*. MMP-induced degradation of fibrinogen supports a plasmin-independent fibrinolysis mechanism. Consequently, degradation of these proteins may be important in inflammation, atherosclerosis, and angiogenesis, all of which are known to be influenced by MMP activity.

The matrix metalloproteinases, MMPs¹ and matrixins, form a family of structurally and functionally related zinc-containing endopeptidases. Together they are able to degrade most of the constituents of the extracellular matrix such as basement membrane, collagens, proteoglycans, fibronectin, and laminin (1). Thus, they are implicated in connective tissue remodeling processes associated with embryonic development, pregnancy, growth, and wound repair (2). The deleterious potential of the MMPs is normally controlled by the endogenous and specific tissue inhibitors of metalloproteinases or the more general nonspecific α_2 -macroglobulin (3). Disturbance of the well balanced equilibrium of MMPs and tissue inhibitors of metalloproteinases results in pathological situations

such as rheumatoid and osteoarthritis, atherosclerosis, tumor growth, metastasis, and fibrosis (4–8). In addition to degradation of extracellular matrix constituents, plasma proteins such as serpins (9) or fibrinogen and cross-linked fibrin (10–12) are also cleaved.

Fibrinogen is a 340-kDa dimeric glycoprotein consisting of a pair of three polypeptide chains A α , B β , and γ that are interconnected by 29 disulfide bonds. The amino termini of these chains are joined together in a central domain that can be isolated as a single fragment from a plasmin digestion of fibrinogen (13). During blood coagulation, fibrinogen participates in both the cellular phase and the fluid phase of blood clot formation (14, 15). Fibrinogen can be converted into an insoluble fibrin clot as a consequence of thrombin-catalyzed removal of fibrinopeptides A (FpA, A α -(20–35))² and B (FpB, B β -(31–44)) from the A α and B β chains (16).

In addition to the ordinary route of thrombin generation (17) via the tissue factor pathway, an alternative route exists that is initiated by the activation of Factor XII (Hageman factor) (18). The activation of Hageman factor to yield active α -Factor XIIa takes place by a single cleavage at ³⁷²R↓V³⁷³ (numbering includes signal peptide) (19). Eventually, cleavage at ³⁵³R↓N³⁵⁴ and ³⁶²R↓L³⁶³ leads to the β -Factor XII (20), which still exhibits full catalytic activity. After several more steps of zymogen activation, this alternative route leads into the ordinary pathway of blood coagulation, terminating in the proteolytic conversion of fibrinogen into fibrin.

The purpose of this work is to examine the role of MMPs in the degradation of fibrinogen and Factor XII. We therefore examined the clotting of MMP-digested fibrinogen to support the idea that some of the biological functions of fibrinogen might be hampered (11). In addition to this, the previously unreported degradation (and inactivation) of Factor XII is shown here. All digestions were subjected to SDS-PAGE followed by automated sequencing to characterize the generated fragments and identify the cleavage sites.

EXPERIMENTAL PROCEDURES

Materials—Lyophilized human fibrinogen (F4883, >95% clottable and essentially plasminogen-free according to the manufacturer) was purchased from Sigma and dissolved to a final concentration of 2 mg/ml. Thrombin and Owren's Veronal buffer (28.4 mM sodium barbital in 125 mM NaCl, pH 7.35) were supplied by DADE (Aguada). EDTA and the synthetic serine proteinase inhibitor Pefabloc® SC (4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride) were delivered from Merck. The chromogenic thrombin substrate S-2302 was supplied by Chromogenix (Moelndal, Sweden). Active recombinant human cdMT1-MMP-(Ile¹¹⁴–Ile³¹⁸) and cdMMP-8-(Met⁸⁰–Gly²⁴²) were prepared as described previously (21, 22). Active human neutrophil gelatinase B (MMP-9) was purified as described previously (23). Active human recombinant cdMT2-MMP was a generous gift from Dr. Horst Will of INVITEK,

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¹ The abbreviations used are: MMP, matrix metalloproteinase; BB-94, batimastat; cd, catalytic domain; FpA, fibrinopeptide A; HPLC, high performance liquid chromatography; MT-MMP, membrane-type MMP; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

² Numbering of amino acids includes signal peptide sequences.

Berlin-Buch, Germany (24). The cdMMP-12 cDNA (base pairs 310–801) was obtained by reverse transcription-polymerase chain reaction from total RNA isolation of placental tissue. The fragment was ligated into the vector pET-11a. pET-11a/cdMMP-12 was transformed into *Escherichia coli* strain BL-21(DE3). Cells were grown to mid-log growth phase (A_{550} , 0.6) and induced with isopropyl- β -D-thiogalactopyranoside for 3 h. Harvested cells were lysed, and the overexpressed protein was isolated as inclusion bodies. The inclusion bodies were dissolved in a urea buffer and dialysed three times against Tris buffer. The protein was purified by affinity chromatography (25). After elution with urea buffer, cdMMP-12 was refolded by dialyzing three times against Tris buffer, pH 7.5. The pro Δ MMP-13 cDNA (base pairs 71–805) was amplified by polymerase chain reaction from the plasmid pEMBL-19 containing the complete cDNA for procollagenase-3 derived by reverse transcription-polymerase chain reaction from total RNA isolated from breast cancer cells (26). The resulting 734-base pair fragment was cloned into the expression vector pET-12b and *E. coli* strain BL21(DE3). The expression and purification of the inclusion bodies were performed as described for cdMMP-12. The protein was purified by Q-Sepharose chromatography and gel filtration with Sephacryl S-100. The isolated pro Δ MMP-13 was activated before use by incubation with 5 mM HgCl_2 for 2 h at 37 °C.

Activity Assays—MMP activity was determined by gelatin zymography (27) and by a continuous assay using MCA peptide ((7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2, 4-dinitrophenyl]-1-2-3-diaminopropionyl)-Ala-Arg-NH₂) as a synthetic substrate (28).

Digestion of Fibrinogen by MMPs—Fibrinogen was incubated with cdMMP-8 in a 1:50 enzyme/substrate molar ratio at 37 °C for different time intervals. In the case of cdMMP-12, MMP-13, and MT1-MMP, a ratio of 1:10 was employed. All reactions were performed in 20 mM Tris-HCl buffer, pH 7.3, containing 100 mM NaCl, 5 mM CaCl_2 , 0.3 mg/ml Pefabloc® SC, and 100 μM ZnCl_2 . Digestions were terminated by addition of 0.1 μl of EDTA (0.1 μmol) and a third part of denaturing buffer (50 mM Tris-HCl pH 6.8, containing 1% SDS, 8 M urea, 30 mM NaCl, 1% 2-mercaptoethanol, and 0.05% bromophenol blue). All reaction products were subjected to SDS-PAGE.

SDS-PAGE—All reaction products were subjected to SDS-PAGE on 10% gels under reducing conditions followed by silver staining (29). The molecular weights of apparent bands were estimated (30).

Determination of Thrombin-induced Clotting of cdMT1-MMP-treated Fibrinogen—The clotting time of cdMT1-MMP-treated fibrinogen was determined with a semiautomatic coagulometer by the functional method of Clauss (31). 1.03 mg of fibrinogen (3.97 nmol) in Owren's Veronal buffer was preincubated for different time intervals at 37 °C with the corresponding amount of MMP-8, MMP-12, MMP-13, and MT1-MMP, respectively. Substrate cleavage was stopped by adding a 10 \times molar excess of BB-94. Clotting was initiated by adding 200 μl of thrombin (90 NIH units/ml) to 200 μl of the pre-warmed (37 °C) fibrinogen sample. A timer was started with addition of the thrombin solution and halted automatically at the point of clotting by a metallic oscillator. Under the conditions used, clotting time depended mainly on the concentration of fibrinogen. The concentration of the remaining clottable fibrinogen was calculated from the clotting time according to a calibration curve that was established with known amounts of untreated fibrinogen.

Amino Acid Sequence Analysis—After enzymatic digestion Factor XII fragments were directly separated by SDS-PAGE on 10% gels and electroblotted to a PVDF membrane (32). Fibrinogen digestion products were first separated by reverse-phase HPLC and then run on 10% gels under reducing and denaturing conditions. After transferring the protein fragments to a PVDF membrane in 50 mM borate/NaOH buffer, pH 9.0, containing 20% methanol, the membrane was stained with Coomassie Brilliant Blue R-250 solution. Protein bands were cut out of the membranes and subjected to automated sequencing on a Knauer gas-phase sequencer with online phenylthiohydantoin-derivative amino acid identification (33).

Reverse-phase HPLC—To identify the MMP cleavage sites in fibrinogen, a sample of the digested protein was subjected to separation by reverse-phase HPLC using a Bakerbond C_{18} -wide pore column. Elution of fibrinogen fragments was performed at ambient temperature using a gradient obtained with 0.1% trifluoroacetic acid (solvent A) and 0.09% trifluoroacetic acid in 80% acetonitrile (solvent B). Column flow rate was 0.6 ml/min. Collected fractions were lyophilized, diluted in denaturing buffer, subjected to SDS-PAGE, and electroblotted to a PVDF membrane.

Functional Factor XII Determination—50 μl of Factor XII (400 $\mu\text{g}/\text{ml}$) was incubated with 5 μl of active MMP (200 $\mu\text{g}/\text{ml}$) in 50 mM Tris-HCl, pH 7.3, containing 100 mM NaCl, 5 mM CaCl_2 , and 100 μM

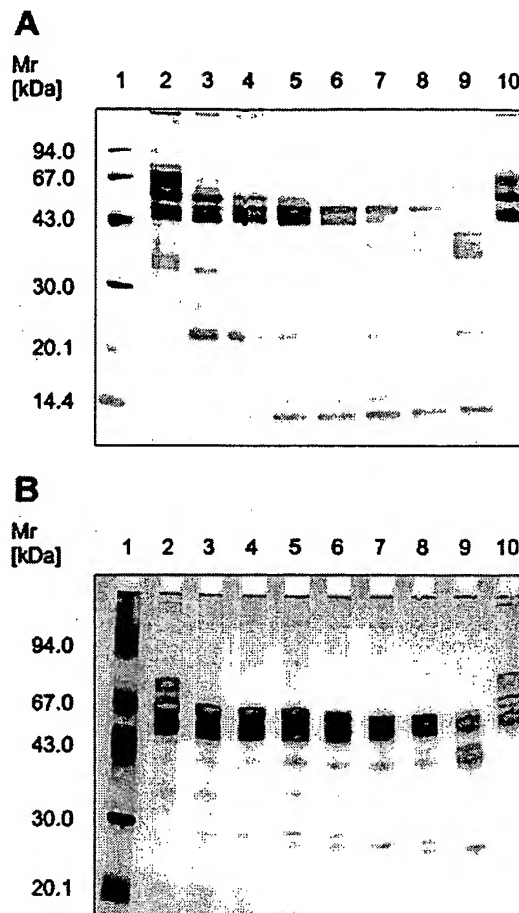


FIG. 1. Time course of fibrinogen digestion by cdMT1-MMP (A) and cdMMP-12 (B). At the indicated time intervals aliquots were taken, and digestion was stopped by adding EDTA and denaturing buffer. Samples were denatured and then electrophoresed on 10% SDS gels. A, fibrinogen was digested by cdMT1-MMP in a 1:10 enzyme/substrate molar ratio for 3 h at 37 °C. Samples were taken at 0, 1 min, 5 min, 15 min, 30 min, 1 h, 2 h, and 3 h. Lane 1, low molecular mass standards; lane 2, fibrinogen at time 0; lanes 3–9, samples taken at indicated time intervals; lane 10, fibrinogen after 3 h of incubation with buffer alone. B, MMP-12-induced fibrinogen degradation was performed with a 1:10 enzyme/substrate molar ratio. The reaction mixture was incubated at 37 °C for up to 2 h. Samples were taken at the indicated time intervals. Lane 1, low molecular mass standards; lanes 2–9, reaction mixture after 0, 2, 4, 8, 15, 30, 60, and 120 min of incubation; lane 10, fibrinogen after 2 h incubated with buffer alone.

ZnCl_2 for 45 and 90 min at 37 °C. After the incubation time was over, 1 μl of batimastat solution (1 mg/ml) was added and incubated for 5 min to inhibit MMP activity. 50 μl of this solution was mixed with 100 μl of S-2302 solution (4 mM) and the absorbance at 405 nm (reference: 490 nm) was measured with an interval of 2 min for 32 min overall. Measurements of the buffer alone, the batimastat solution, the active MMP alone, and the Factor XII solution without active MMP were taken as controls.

RESULTS

MMP-induced Degradation of Fibrinogen—We examined the catabolic activities of several MMPs on fibrinogen. Samples of digestion were taken at different time intervals, heat-treated in denaturing buffer, and subjected to SDS-gel electrophoresis. The time course of fibrinogen degradation after incubation with cdMT1-MMP is shown in Fig. 1A. Under reducing conditions in SDS gels, fibrinogen was separated into its subunits. The α , β , and γ chains could be distinguished. The catalytic domain of MT1-MMP digested fibrinogen rapidly and extensively when an enzyme/substrate molar ratio of 1:10 was employed. cdMT1-

TABLE I
Depiction of the identified NH₂-terminal sequences of HPLC fractions
Fibrinogen was subjected to MMP-cleavage, and the resulting fragments were isolated. The amino acid sequences were determined by automated Edman degradation.

Enzyme	kDa	NH ₂ -terminal sequence ^a	Origin
MMP-8	68	²⁰ ADSGEGD	α-chain
	32	²⁰ ADSGEGD	α-chain
	30	²⁰ ADSGEGD	α-chain
	13	⁴⁴² LRTGKEKV	α-chain
	12	⁴⁴² LRTGKEKV	α-chain
MMP-12	10	⁴⁴² LRTGKEKV	α-chain
	50	²⁰ ADSGEGD	α-chain
	30	²⁰ ADSGEGD	α-chain
	25	²⁰ ADSGEGD	α-chain
	15	⁵⁴⁰ FVSETESRG	α-chain
MMP-13	13	⁴³³ LVTSGDK	α-chain
	12	⁵⁴⁰ FVSETESRG	α-chain
	45	²⁷ YVATRDN	γ-chain
	35	ND ^b	
	32	²⁰ ADSGEGD	α-chain
MMP-14	28	¹²⁴ RNSVDXLNXN	β-chain
	16	ND	
	14	⁴⁴² LRTGKEKV	α-chain
	45	²⁷ YVATRDN	γ-chain
	38	¹⁰⁵ XDAATLKSR	γ-chain
	35	⁹² LTYNPDES	γ-chain
	15	¹⁰⁵ LTNIXEXL	α-chain
	13	⁴³³ LVTSGDK	α-chain
	6	¹¹⁷ FXSANNRD	α-chain

^a The numbering of amino acids of all proteins includes signal peptide sequence.

^b Not determined.

MMP treatment of fibrinogen resulted in the complete disappearance of fibrinogen α-chain after a 1-min incubation. In turn, several smaller α-chain fragments with molecular masses of 15, 13, and 6 kDa became apparent and could be identified. During fibrinogen digestion, progressive decreases of the 56-kDa β-chain and the 47-kDa γ-chain were observed, leading to γ-chain fragments sized at 38 and 35 kDa, respectively. These fragments underwent further degradation to yield unidentified smaller digestion products. Thus, after 30 min of incubation no remaining β-chain could be visualized, and after 2 h the γ-chain was also completely cleaved. With MMP-12, quick disappearance of the fibrinogen α-chain could also be observed. No corresponding band was visible after a 2-min incubation. Fig. 1B shows that the bands indicating β- and γ-chains were diminished to a lesser extent than observed with MMP-14.

Consistent with the observation of fibrinogen fragmentation, the generation of lower molecular mass products was noted with time leading to a set of bands. Of these, six could be identified by means of amino acid sequence analysis (Table I). Generally, we observed fast degradation of the fibrinogen α-chain by MMP-8, MMP-12, MMP-13, and MT1-MMP. At longer incubation times, degradation of β- and γ-chains were also noted. The identified amino termini of the generated fibrinogen fragments are summarized in Table I.

Characterization of Fibrinogen Fragments—To identify the fibrinogen fragments generated by cdMMP-13 and cdMMP-14, respectively, separation by reverse-phase HPLC was performed. Fig. 2 shows a typical HPLC profile for MT1-MMP-digested fibrinogen. We analyzed the homogeneity of the various peak fractions by subjecting a sample of each fraction to SDS-PAGE under reducing conditions. Fractions containing more than one fragment were separated by SDS-PAGE and electroblotted onto a PVDF membrane.

Effect of MMPs on the Thrombin-induced Clotting of Fibrinogen—To further characterize the effect of fibrinogen degradation by MMPs, the clotting of digested fibrinogen was measured

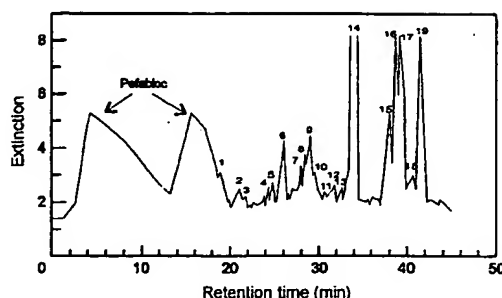


Fig. 2. HPLC elution profile of fibrinogen digestion products. After 2 h of incubation with cdMT1-MMP and cdMMP-13, respectively, the reaction mixture was injected onto the column. To achieve a satisfactory separation of the generated fragments, an acetonitrile gradient of 0–60% B in 35 min, 60–100% B in 40 min, (see "Experimental Procedures") was employed. The flow rate was 0.6 ml/min. 19 fractions (see corresponding numbering) were collected, lyophilized, and analyzed by SDS-PAGE.

by the functional method of Clauss (31). Fibrinogen was incubated with cdMMP-8, cdMMP-13, cdMMP-12, and cdMMP-14, respectively, for up to 2 h, and after stopping the fibrinogen degradation by adding a 10-fold molar excess of BB-94, the clotting capability of fibrinogen was measured. Fig. 3 shows the amount of clottable fibrinogen measured at different points of time. The individual fibrinogen concentrations were calculated from standard dose-response curves of fibrinogen concentrations versus clotting time. After an 8-min incubation with cdMT1-MMP, remaining fibrinogen clotting activity was already reduced to approximately 68%. Moreover, the clotting was reduced to an even greater extent when fibrinogen was incubated with MMP-8, MMP-12, or MMP-13, respectively. These results indicate a markedly impaired coagulant activity of MMP-treated fibrinogen compared with normal fibrinogen. Similar results were obtained by measuring clotting of MMP-8, MMP-12, or MMP-13-treated fibrinogen (see Fig. 3).

Degradation of Hageman Factor by MMPs—To investigate the cleavage of Factor XII by MMP-13, reagents were incubated at 37 °C for up to 3 h (see Fig. 4A). The proteolytic cleavages resulted in breakdown of Factor XII into several fragments within minutes. With SDS-PAGE, after 4 min of incubation, two intermediate bands became apparent at 45 and 30 kDa, respectively. Prolonged incubation led to further degradation and disappearance of these fragments, and bands of lower molecular weight became visible (27 and 22 kDa, respectively). Amino-terminal sequence identification of these fragments showed that cleavage occurred at the beginning of the type II fibronectin-like and the epidermal growth factor-like domains. Moreover, a cleavage within the catalytic region was identified four residues downstream of the kallikrein cleavage site that leads to the activation of Factor XII. It is noteworthy that the fragment Leu³⁷⁷-Ser⁶¹⁵ generated by MMP-12, MMP-13, and MT1-MMP did not show any catalytic activity against the chromogenic substrate S-2302.

In addition to this, we have discovered that Factor XII cannot be activated by kallikrein after MMP-induced cleavage. This was shown by a separate experiment in which latent Factor XII was subjected to treatment by MMPs and tested for activation with the synthetic substrate S-2302. After addition of kallikrein, the control of untreated Factor XII yielded the expected cleavage of the chromogenic substrate whereas MMP-digested Hageman factor showed no activity.

Cleavage of Hageman factor by cdMMP-12 is as as yet unknown capability of this matrix metalloproteinase. The time course of this degradation is shown in Fig. 4B. The amino termini were also identified as His²⁹ and Leu³⁷⁷ after cleavage by MMP-13. The amino terminus Tyr³¹ at the beginning of the

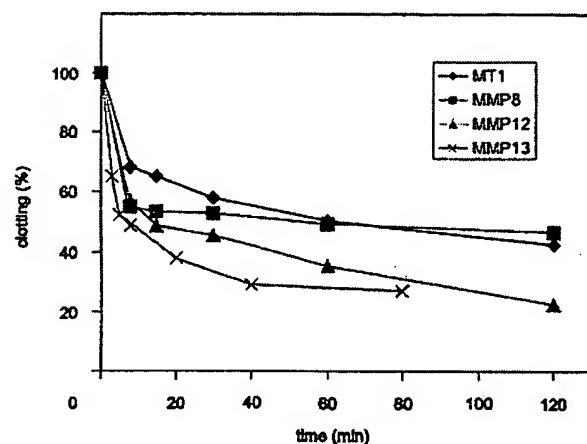


FIG. 3. Effect of MMPs on the thrombin-induced clotting of fibrinogen. Clotting was expressed as the percentage of clottable protein after incubation with cdMMP-8, cdMMP-12, cdMMP-13, and cdMT1-MMP, respectively, in a time-dependent manner. The clotting of untreated fibrinogen (control, time point: 0 min) was set to 100%. Values are the mean of two determinations, and the standard deviations are <10%.

type II fibronectin-like domain was identified as a yet unknown cleavage site generated by cdMMP-12. Moreover, the specificity for the cleavage site of human metalloelastase (HME) at residue Leu³⁸⁰ at the beginning of the catalytic region was also unknown. Cleavage of Hageman factor by MMP-14 generates fragments with approximate molecular masses of 50, 45, 33, 30, and 12 kDa. The identified amino terminus Leu³⁷⁷ was the same as that obtained with MMP-12 and MMP-13. The amino-terminal residue Leu³⁵¹ was also generated by MMP-14 (Table 2).

DISCUSSION

Proteolytic Degradation of Fibrinogen—The data presented in this work demonstrate the fibrinogenolytic activities of the catalytic domains of MMP-8, MMP-12, MMP-13, and MT1-MMP. All enzymes degrade predominantly the A α -chain of fibrinogen and at longer incubation times the B β -chain and γ -chain as well. The structural alterations in fibrinogen caused by different concentrations of the employed matrix metalloproteinases were analyzed by electrophoresis of treated fibrinogen in polyacrylamide gels under denaturing and reducing conditions. We found that the MMP-treated fibrinogen was rapidly degraded to lower molecular mass intermediates (Fig. 1). At the earliest time point (1 min), the A α -chains were almost completely cleaved whereas the B β - and γ -chains were apparently unaffected. When the incubation time was extended to 3 h, the staining intensities of the β - and γ -chains were also diminished. Most of the generated fibrinogen fragments were identified by amino-terminal sequencing (see Table 1). Sequencing data revealed that these fragments resulted mainly from cleavages in the A α - and γ -chains of fibrinogen (Fig. 5). However, we were not able to determine the amino termini of all generated fragments. Amino acid sequence analysis of yet unidentified digestion products is currently being performed.

Effect of MMPs on the Thrombin-induced Clotting of Fibrinogen—To determine whether MMP treatment of fibrinogen influences thrombin-catalyzed fibrin polymerization, we measured the clotting time of fibrinogen after digestion with cdMMP-8, cdMMP-12, cdMMP-13, and cdMT1-MMP. The clotting time reflected the amount of intact fibrinogen remaining after proteolytic attack by the individual matrixin.

We have proven that the degradation of fibrinogen by MMPs resulted in a loss of fibrinogen function. Under the applied

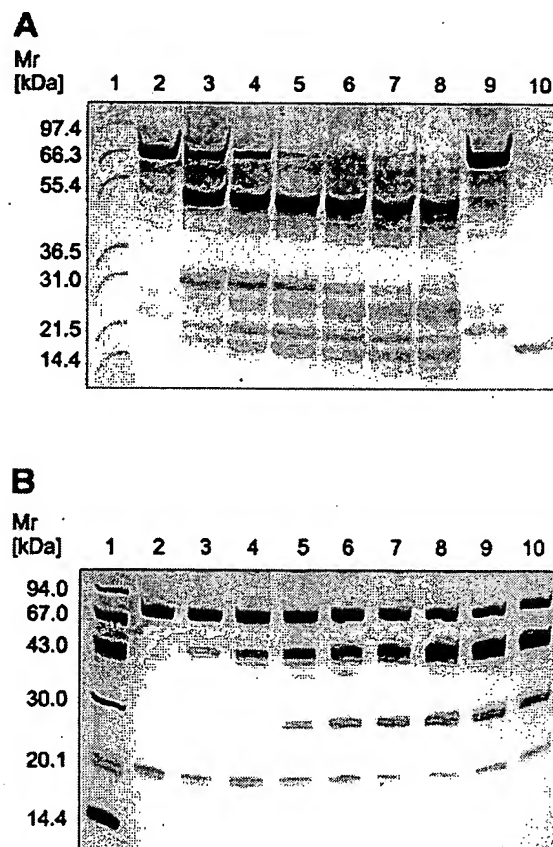


FIG. 4. Time course of Factor XII digestion by cdMMP-13 (A) and MMP-12 (B). At the indicated time intervals aliquots were taken and the digestion was stopped by adding EDTA and denaturing buffer. Samples were then electrophoresed on 10% SDS gels. A, Factor XII was digested by cdMMP-13 in a 1:30 enzyme/substrate molar ratio for 3 h at 37 °C. Samples were taken at 0, 4, 8, 16, 32, 64, and 128 min. Lane 1, low molecular mass standards; lanes 2–8, samples taken at indicated time intervals; lane 9, Factor XII after 3 h of incubation with buffer alone; and lane 10, active cdMMP-13 (control). B, digestion of Hageman factor by MMP-12 was done with an enzyme to substrate ratio of 1:10 for up to 2 h at 37 °C. Samples were taken at 0, 2, 4, 8, 15, 30, 60, 90, and 120 min. Lane 1, low molecular mass standards; lanes 2–10, samples taken at the indicated time intervals.

TABLE II
Identified NH₂-terminal sequences of MMP-cleaved Hageman factor
Factor XII was degraded by MMPs and the generated NH₂ termini were isolated and identified by Edman degradation.

Enzyme	kDa	NH ₂ -terminal sequence ^a	Origin
MMP-12	43	²⁹ HKYKAEHT	type II FN
	43	³¹ YKAEHTV	type II FN
	32	³⁵¹ LTRNGPL	catalytic region
	30	³⁷⁷ LVALRGAH	catalytic region
MMP-13	20	³⁸⁰ LRGAHPY	catalytic region
	45	²⁹ HKYKAXEH	type II FN
	43	⁹⁴ VKDHXSKEH	growth factor
	30	³⁷⁷ LVAXRGAH	catalytic region
MMP-14	27	³⁷⁷ LVAXRGAH	catalytic region
	70	IXXEAXKEH	NH ₂ -terminus
	45	ND ^b	
	33	³⁵¹ LTRNGPL	catalytic region
	30	³⁷⁷ LVALRGA	catalytic region

^a The numbering of amino acids of all proteins includes signal peptide sequence.

^b Not determined.

conditions, fibrinogen showed dramatically impaired clotting ability. In this context, we would like to mention that we were not able to determine correct clotting times of fibrinogen sam-

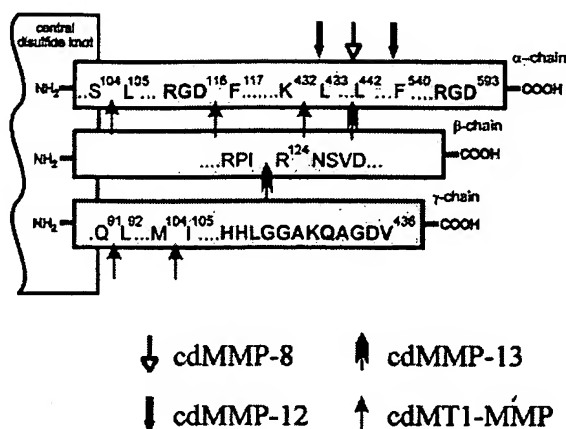


FIG. 5. Simplified model of the fibrinogen molecule and its cleavage sites for cdMT1-MMP. Only one-half of the symmetrical molecule is shown. The locations of the putative adhesive sites identified for $\alpha_{IIb}\beta_3$ integrins are indicated in **bold letters**. These include RGD both at the amino and carboxyl termini of the α -chain and the dodecapeptide sequence HHLGGAKQAGDV at the carboxyl terminus of the γ -chain, respectively (53). The cleavage sites for the individual MMPs are indicated by corresponding arrows.

ples digested for times shorter than 5 min because of a delayed termination of MMP-mediated fibrinogen degradation by addition of BB-94. Thus, the determined concentrations of functional fibrinogen in the early stage of digestion appear to be too low. Nevertheless, the obtained data show that treatment of fibrinogen with matrixins prevents the self-assembly of large protofibrils and fibers. The clotting of fibrinogen was studied with *in vitro* assays because the catalytic domain of MT1-MMP was not enzymatically active in stabilized human plasma due to the presence of citrate or EDTA. The physiological significance of our findings refers to the obviously altered coagulant properties of digested fibrinogen. This fact may influence a number of cellular events including tumor growth, wound healing, and cell-attachment. Fibrinogen mediates cellular adhesion of a number of different cell types including thrombocytes, endothelial cells, and tumor cells.

Fibrinogen contains RGD sequences both at the amino and carboxyl termini of the α -chain. These sequences as well as the carboxyl terminus of the γ -chain bind to the platelet glycoprotein II_b-III_a (integrin $\alpha_{IIb}\beta_3$) (34, 35). During incubation of fibrinogen with cdMT1-MMP, both α -chain RGD sequences were removed as well as the γ -chain dodecapeptide sequence (see Fig. 4). With MMP-8, MMP-12, and MMP-13, at least one of the RGD motifs is released. These cleaved fibrinogen fragments may still be recognized by and bound to glycoprotein II_b-III_a. Nevertheless, platelet aggregation may be inhibited because the binding peptides are no longer interconnected.

The data presented in this study show for the first time the degradation of Factor XII of the blood clotting system by matrix metalloproteinases. MMP-12, MMP-13, and MMP-14 cleave at Gly³⁷⁶ ↓ Leu³⁷⁷. This cleavage site lies four residues downstream of the kallikrein cleavage site. Conversion of latent Hageman factor into its active form, Factor XIIa, takes place with cleavage of the Arg³⁷² ↓ Val³⁷³ peptide bond (19, 36). However, no activity of Factor XII can be observed after MMP-induced cleavage. Moreover, MMP-treated latent Hageman factor cannot be activated by kallikrein (results not shown). It is therefore conceivable that the activation of Factor XII requires the kallikrein-generated terminal positive charge of Val³⁷³ to establish a stabilizing salt bridge as known from trypsin (37, 38). Fig. 6 displays the different cleavages of Hageman factor by MMP-12, MMP-13, and MT1-MMP in compari-

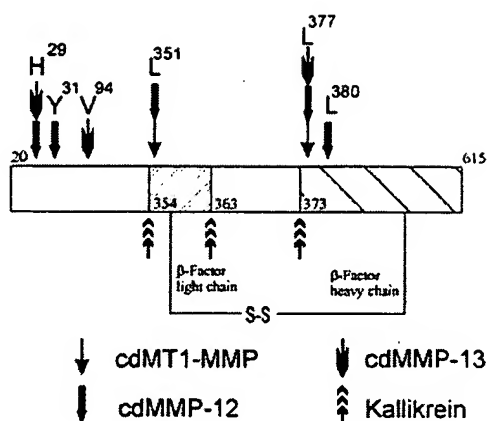


FIG. 6. Block diagram of the Hageman factor molecule and cleavage sites for MMPs. Cleavage of MMP-12, -13, and -14 are compared with the naturally occurring cleavages by kallikrein leading to α - and β -Factor XIIa, respectively. The cleavage sites for the individual proteinases are indicated by the corresponding arrows.

son to the naturally occurring cleavages by kallikrein. The effect of Factor XII cleavage by MMPs *in vivo* remains to be clarified because the ordinary route of thrombin generation takes place via the tissue factor pathway and is therefore independent of Hageman factor (18).

The importance of plasma protein cleavage by MMPs has to be further investigated, especially in tumor cells and endothelial cells that express high levels of MMPs. In 1995, the expression of MT1-MMP mRNA transcript in vascular endothelial cells was demonstrated (39). This led to the speculation that this enzyme interacts not only with constituents of the extracellular matrix but also with blood components (12). Moreover, the enzyme was identified by polymerase chain reaction screening in human monocytes isolated from peripheral blood (40). MMP-8 has also been shown to have a possible influence on plasma proteins after secretion from polymorphonuclear leucocytes (9). Several cell lines, e.g. T-lymphocytes (41), macrophages, and endothelial cells (42), are known to express collagenase-3, which in turn can act on plasma proteins. The human metalloelastase is known to interact with several other plasma proteins (43, 44).

There is evidence for the successive release of MMPs in buffycoat-depleted red cell concentrates of blood donors during storage (45, 46). It is anticipated that these matrixins may also have the same mode of action *in vivo*. Taken together, these findings are consistent with an interaction of MMPs with blood components, especially with plasma proteins of the clotting system.

Consequences—The important role of matrixins in tumor invasion is suggested by a large number of correlation studies demonstrating a direct relationship between increased expression of proteases in tumor tissues and their invasive and metastatic behavior (47). Recently, several groups have shown the degradation of several plasma proteins by some MMPs. Our experiments clearly support the findings that proteolytic activity of matrixins is not restricted to extracellular matrix components. Moreover, the spectrum for MMPs is broader, now including proteins involved in hemostasis like fibrinogen (Fig. 5 and Refs. 10 and 11), Factor XII (Fig. 6), plasminogen (48), and plasmin (43). These data suggest that MMPs in interaction with plasma proteins may play a role in the control of coagulation. In this work, we demonstrate a possible down-regulation of thrombotic potential caused by the impact of MMP on proteins participating in coagulation processes. This is an important finding that goes beyond physiological and pathological

conditions. Moreover, with respect to the successive release of MMPs from residual leukocytes during storage (9, 45, 46, 49), degradation of plasma proteins by MMP-8, MMP-9, MMP-12, and MMP-13 may occur when administering red cell concentrates to patients.

Comments—Our experiments were conducted with carboxyl-terminal truncated enzymes. It had been suggested that the substrate specificity of matrixins is attributed to the presence of the hemopexin-like domain at the carboxyl terminus of most of the MMPs (50). However, D'Ortho *et al.* (51) compared the substrate specificity of the catalytic domain of MT1-MMP with that of a variant of MT1-MMP containing the catalytic domain and the carboxyl-terminal hemopexin-like domain. These authors noted that both enzyme variants degraded the same substrates with comparable efficiency with the exception of the triple-helical collagens I and III. Cleavage of collagens required the presence of both the catalytic and the hemopexin-like domain. This matrixin domain appeared to have no role in the proteolytic specificity directed toward other substrates. We made the same observation with MMP-8, MMP-9 (52), and MMP-14 (results not shown).

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